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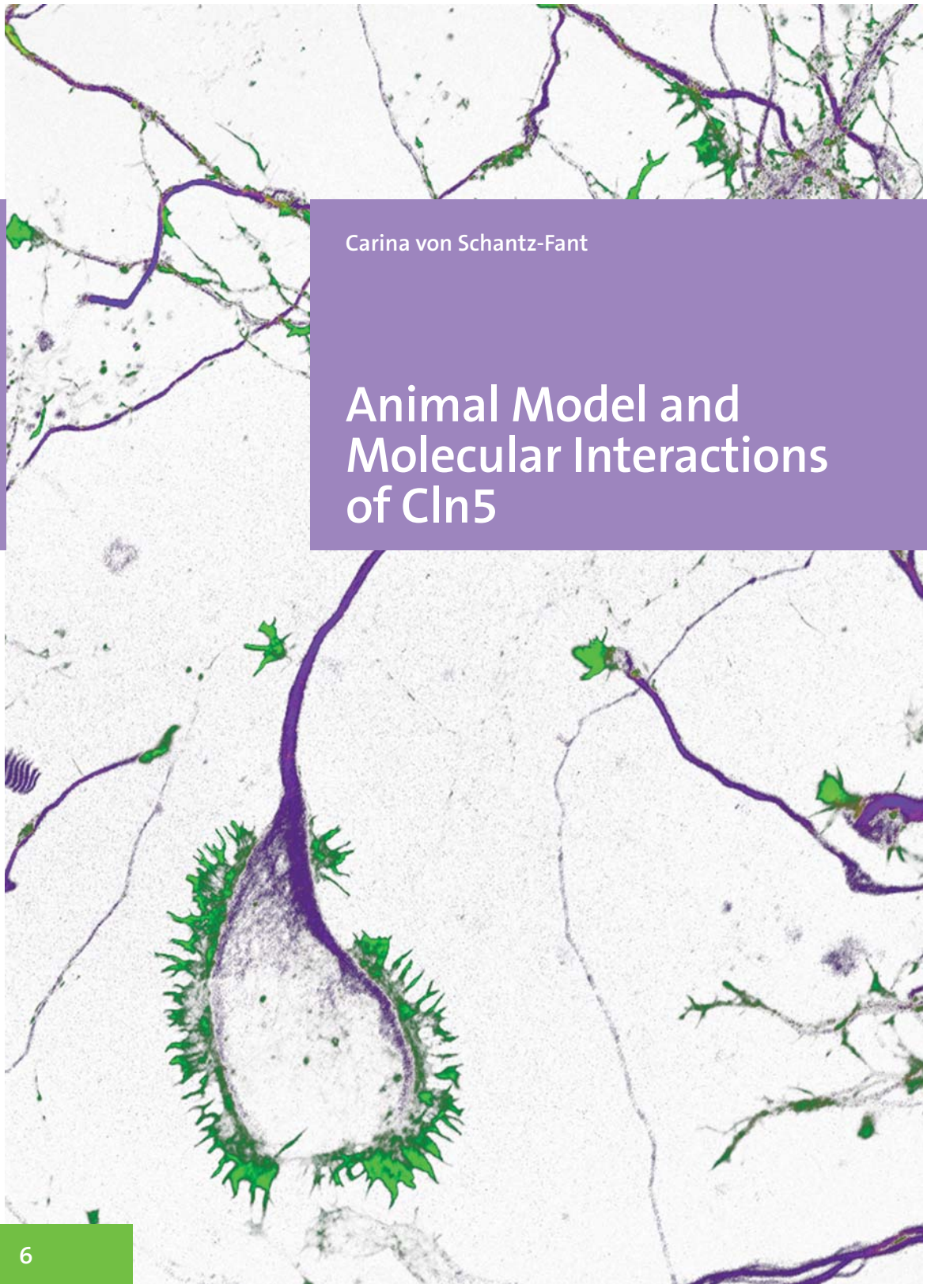

RESEARCH

Carina von Schantz-Fant

Animal Model and Molecular Interactions of Cln5

Carina von Schantz-Fant

"ANIMAL MODEL AND MOLECULAR INTERACTIONS OF CLN5"

ACADEMIC DISSERTATION

To be presented with the permission of the Medical Faculty of the University of Helsinki, for public examination in the large lecture hall, Haartman Institute, on April 3rd, 2009, at 12 noon.

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”If we knew what we were doing we wouldn’t call it research”

-Albert Einstein

To my family

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ABSTRACT

Neuronal ceroid lipofuscinoses (NCLs) are a family of inherited pediatric neurodegenerative disorders, with an incidence of 1:12 500 in the US and Scandinavian countries and 1:100 000 worldwide. The major hallmarks of NCLs include retinal degeneration, death of selective neuronal populations and accumulation of autofluorscent ceroid-lipopigments. The clinical manifestations are generally similar in all forms.

The Finnish variant late infantile neuronal ceroid lipofuscinosis ($vLINCL_{Fin}$) is a form of NCL, especially enriched in the Finnish population. The clinical symptoms include motor clumsiness, progressive retinal degeneration, motor and mental deterioration, myoclonia and seizures.

The first aim of this thesis was to analyse the brain pathology of $vLINCL_{Fin}$ utilising the novel *Cln5*-/- mouse model. The *Cln5*-/- mouse presented with the classical hallmarks of the vLINCLFin pathology, including autofluorescence and typical ultrastructure of the storage deposits in brains of the affected mice and therefore, proved to be an excellent model to study $vLINCL_{Fin}$. Gene expression profiling of the brains of already symptomatic *Cln5*-/- mice revealed that inflammation, neurodegeneration and defects in myelinization are the major characteristics of the later stages of the disease.

Histological characterization of the brain pathology confirmed that the thalamocortical system is affected in *Cln5*-/- mice, being consistent with the findings from other NCL mouse models. Whereas the brain pathology in all other analyzed NCL mice initiate in the thalamus and spread only months later to the cortex, we observed that the sequence of events is uniquely reversed in *Cln5*-/- mice; the neurodegeneration begins in the cortex and becomes evident in the thalamus only months later. We could also show that even though neurodegeneration is inititated in the cortex, reactive gliosis and loss of myelin are evident in specific nuclei of the thalamus already in the 1 month old brain.

To obtain a deeper insight into the disturbed metabolic pathways early in the course of the disease, we performed gene expression profiling of the mouse brains. We substantiated these findings with immunohistological analyses, and could

demonstrate that cytoskeleton and myelin were affected in *Cln5-/-* mice. Comparison of gene expression profiling of two NCL mouse models further highlighted that the *Cln5-/-* and *Cln1-/-* mice share a common defective pathway, leading to disturbances in the neuronal growth cone and cytoskeleton.

Encouraged by the first evidence of common pathogenetic mechanisms behind two forms of NCL, we systematically analyzed the molecular interactions of NCL-proteins and observed that Cln5 and Cln1/Ppt1 proteins interact with each other. Furthermore, we demonstrated that Cln5 and Cln1/Ppt1 share an interaction partner, the F1-ATP synthase, potentially linking both $LINCL_{FIN}$ and INCL diseases to disturbed lipid metabolism. In addition, Cln5 was also shown to interact with other NCL proteins; Cln2, Cln3, Cln6 and Cln8 implicating a central role for Cln5 in the NCL pathophysiology.

This study is the first to describe the brain pathology and gene expression changes in the *Cln5-/-* mouse. Together the findings presented in this thesis represent novel information of the disease processes and the molecular mechanisms behind $vLINCL_{Fin}$ and have highlighted that the relatively rare Finnish CLN5/$vLINCL_{Fin}$ forms a very important model to analyze the pathophysiology of NCL diseases, protein interactome and effects between genes and proteins leading to neurodegeneration in general.

Keywords: neurodegeneration, lysosomal storage disease, neuronal ceroid lipofuscinosis, gene expression profiling

CONTENTS

ABBREVIATIONS

AIF	apoptosis-inducing factor
ANCL	adult neuronal ceroid lipofuscinosis (CLN4)
apoA-I	apolipoprotein A-I
BBB	blood brain barrier
Cap-1	adenylate cyclase-associated protein 1
cb	cerebellum
cc	corpus callosum
Ccl21a	chemokine (C-C motif) ligand 21 a gene
CLN5/Cln5	CLN5 gene/protein
CNP	2′,3′-cyclic nucleotide 3′-phosphodiesterase gene
CNS	central nervous system
CONCL	congenital neuronal ceroid lipofuscinosis (CLN10)
COS-1 cells	African green monkey kidney cells
CTSD	Cathepsin D gene
Ddx6	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 gene
DNA	deoxyribonucleic acid
Drpla	dentatorubral-pallidoluysian atrophy protein
EEG	electroencephalography
EPMR	Progressive epilepsy with mental retardation (CLN8)
ER	endoplasmic reticulum
ERG	electroretinogram
ERGIC	ER and the ER-Golgi intermediate compartment
ES	Embryonic stemcells
GDNF	glial cell line-derived neurotrophic factor gene
GFAP	glial fibrillary acid protein
GO	Gene Ontology
Gprc5b	G-protein-coupled receptor family C, group 5, member B gene
GROD	granular osmiophilic deposit
HeLa cells	human cervical tumour cells

HIV	Human immunodeficiency virus
IF	immunofluorescence
IGFR	insulin-like growth factor receptor gene
INCL	infantile neuronal ceroid lipofuscinosis (CLN1)
JNCL	juvenile neuronal ceroid lipofuscinosis (CLN3)
kDa	kilodalton(s)
KH	K homology
Kif5c	kinesin family member 5C gene
Lent	late entorhinal cortex of thalamus
LFB	luxol fast blue stain
LGNd	dorsal lateral geniculate nucleus of thalamus
LINCL	late infantile neuronal ceroid lipofuscinosis (CLN2)
LSD	lysosomal storage disease
M1	primary motor cortex
M6-P	mannose 6-phosphate
MAG	myelin-associated glycoprotein
MEG	magnetoencephaography
MBP	myelin basic protein
MFS	major facilitator superfamily
MGN	medial geniculate nucleus of thalamus
MHC	major histocompatibility complex
Mnd	motor neuron degeneration
MOG	myelin-oligodendrocyte glycoprotein
MPR	mannose 6-phosphate receptor
mRNA	messenger RNA
MT	microtubule
NCL	Neuronal ceroid lipofuscinosis
NGF	nerve growth factor
NMD	Nonsense-mediated decay
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis

PCD	Programmed cell death
PCR	polymerase chain reaction
PLP	proteolipid protein
PLTP	plasma phospholipid protein
PPT1/Ppt1	palmitoyl protein thioesterase 1 protein (CLN1)
Ptprf	protein tyrosine phosphatate receptor type F gene
Qk	quaking gene
RNA	ribonucleic acid
S1BF	somatosensory barrelfield cortex
SAP, Saposin	sphingolipid activating protein
SDS	sodium dodecyl sulphate
SPF	pathogen free facilities
STAR	signal transduction and activator of RNA
TGN	trans-Golgi network
Tia 1	cytotoxic granule-associated RNA binding protein 1
TLC famliy	Tram-Lag1p-CLN8 family of genes
tLINCL	Turkish variant form of late infantile neuronal ceroid lipofuscinosis
TNF-a	tumor necrosis factor alpha gene
TNFR1	tumour necrosis factor receptor 1 gene
TPP1	tripeptidyl peptidase I gene
UPR	unfolded protein response
V1	primary visual cortex
vLINCL	variant form of late infantile neuronal ceroid lipofuscinosis (CLN6)
vLINCLFin	Finnish variant form of late infantile neuronal ceroid lipofuscinosis (CLN5)
VPM/VPL	ventral posterior thalamic nucleus
WB	western blot
wt	wild type
Xist	inactive X specific transcripts gene

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I. Kopra O*, Vesa J*, **von Schantz C**, Manninen T, Minye H, , Fabritius A-L, Rapola J, van Diggelen O.P, Saarela J, Jalanko A, Peltonen L. (2004) A mouse model for Finnish variant late infantile neuronal ceroid lipofuscinosis, CLN5, reveals neuropathology associated with early aging. Hum Mol Genet. Dec 1;13(23):2893-906.

II. **von Schantz C**, Saharinen J, Kopra O, Cooper JD, Gentile M, Hovatta I, Peltonen L, Jalanko A. (2008) Brain gene expression profiles of Cln1 and Cln5 deficient mice unravel common molecular pathways underlying neuronal degeneration in NCL diseases. BMC Genomics. Mar 28;9:146.

III. **von Schantz C**, Kielar C*, Hansen SN*, Pontikis CC, Alexander NA, Kopra O, Jalanko A, Cooper JD (2009). Progressive thalamocortical neuron loss in Cln5 deficient mice: distinct effects in Finnish variant late infantile NCL. *In press*. Neurobiol.Dis

IV. Lyly A*, **von Schantz C***, Heine C, Schmiedt M-Li, Sipilä T, Jalanko A, Kyttälä A. (2009) Neuronal ceroid lipofuscinosis protein CLN5 is a central player in the NCL protein interactome and is connected to PPT1/CLN1 via intracellular transport and F1-ATPase interaction. *Submitted.*

* These authors contributed equally to this work

IV previously used in the thesis work of Annina Lyly

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1 INTRODUCTION

Neurodegenerative disorders are a group of central nervous system disorders characterized by progressive death of brain cells. They comprise a large and increasing share of the global burden of disease. The World Health Organisation (WHO) has reported that although neurological diseases and mental illnesses are responsible for about 1% of deaths, they account for 11% of the disease burden (Oliver et al., 2005).

Lysosomal storage disorders (LSDs) are a group of hereditary metabolic diseases, caused by defects in lysosomal proteins, or proteins affecting the function of lysosomes. The majority of lysosomal storage disorders lead to neurodegeneration and are therefore classified as neurodegenerative disorders. Neuronal ceroid lipofuscinoses (NCLs) are a group of lysosomal storage disorders, mostly affecting children. They are characterized by accumulation of lipopigments in the cells of the body and by progressive death of neurons. The NCLs are grouped according to the age of onset into 10 different subtypes. Yet all lead to death, usually within the second decade of life.

The Finnish variant late infantile neuronal ceroid lipofuscinosis ($vLINCL_{Fin}$) is, although rare, especially enriched in the Finnish population. The disease manifests around the age of 4-7 years, with motor clumsiness and loss of vision being the first of many symptoms.

A great deal of progress has been made in the $vLINCL_{Fin}$ research during the past decade. However, the brain is a difficult organ to study and therefore much of the research has been based on brain material from already deceased patients. Results gained from these studies are able to shed light on disease processes at the end stages of the disease. However, to understand the initial patophysiology of the disease and to be able to develop therapies, it is crucial to study the disease process already at presymptomatic stages.

The use of mouse models allows researchers to investigate diseases in ways that would not be possible in human patients. They are invaluable for studying pathophysiology and also for testing new approaches to therapy. In this thesis, the aim was to investigate the brain pathology of $vLINCL_{Fin}$ utilising the novel *Cln5*-/- mouse model.

2 REVIEW OF THE LITERATURE

2.1 The mouse as a disease model

Diseases are generally caused by defective cellular proteins and one essential goal of biological science is to resolve their physiological function. This is an overwhelming task, since every protein functions together with several others in the living cell. One approach is to use *in vitro* systems, such as cell cultures, to remove the protein of interest in order to study the consequences. However, a protein may have different functions in different cell types. Therefore, studying processes only in specific cell types in a controlled environment outside of a living organism, can lead to results which do not correspond to the situation that arises in a living organism.

Revealing the physiological function of defective disease causing protein and characterizing their pathological events can enable the development of therapies for disease. There are however, many ethical issues concerning procedures performed on humans. As a result animal models have been generated to allow researchers to investigate disease states in ways which would be inaccessible in a human patient.

Genetically altered mouse models can be used to study physiological functions of particular proteins as well as to model human diseases. Such mouse models have been used in the fields of immunology and developmental biology with great success for two decades now. These mouse models can be studied in numerous ways, ranging from biochemistry to cell biology, systems biology and behaviour. Within the past decade, the use of mouse-models to study diseases of the central nervous system has increased rapidly (Picciotto & Wickman, 1998).

Genetically altered mouse models can be created in several ways. ***Transgenic mice*** are created by the addition of an extra gene in the mouse genome. This method is mostly used for models of dominantly inherited diseases, as the inserted mutated gene gives rise to a phenotype regardless of the two endogenous copies of the gene. ***Knock-out mice*** are produced by removing or altering DNA sequences in order to destroy a functioning gene in the mouse genome. This technique is used mostly when studying recessive diseases. Knock-out mice are generated by modifying a DNA sequence, which is then inserted into a cloned copy of the selected gene, by standard recombinant DNA technology. After that, the modification is transferred, by homologous recombination, to the related genomic locus in embryonic stem cells (ES cells). The ES cell lines carrying the desired mutation are then selected and injected into mouse blastocysts. The blastocysts are transferred into foster mothers,

generating chimeric mice that are able of transmitting the modified genetic locus to their offspring (Capecchi, 2001; Capecchi 1989). ***Knock-in mice*** can be used to investigate specific disease mutations. In this case the gene is neither overexpressed nor completely deleted. Manipulation of the endogenous gene can be achieved so as to mimic specific mutations occurring in human disorders. This allows the analysis of how the actual mutated protein product functions in the cell and contributes to the disease phenotype. If a gene is essential for the development, then destroying its function can lead to lethality during embryonic stages or at birth. In such cases, ***conditional mutant mice*** can be generated. Here the expression of the mutated gene can be regulated, by expressing or deleting them at different times of development or in different tissues (Hafezparast et al., 2002; Picciotto & Wickman, 1998).

2.1.1 Benefits of mice

As mammals, mice and humans are alike in many ways; sharing a large number of genes and biochemical pathways. Both humans and mice have approximately 20,000 genes (Carninci & Hayashizaki 2007) and it has been estimated that 99% of human genes have counterparts in the mouse genome (Tecott, 2003). We furthermore have the same diseases as well as common anatomical features. Further, analysis of the behaviour of the mouse, including its social and emotional activity enables modelling of even complex neurological and psychiatric disorders (Oliver & Davies, 2005).

There are many experiments that can be performed in mice which would not be possible to perform in humans, due to ethical concerns. In the case of neurodegenerative diseases, only tissues from the end stage of the disease are generally available from human subjects. However, early stage tissues can be obtained from mice allowing for the study of early symptomotology that manifest before neurodegeneration. Further, the mouse embryo can be studied to elucidate congenital or perinatal diseases. Additionally, new therapies, including gene therapies can be tested in mice before trials can be concidered in humans (Hafezparast et al., 2002; Oliver & Davies, 2005).

Mice are common model animals because of their availability, size, low cost, ease of handling, and fast reproduction rate. They have a short gestation period, an early puberty and a short oestrus cycle. They usually produce large litters, and their mating as well as their environment can be strictly controlled (Willis-Owen & Flint, 2006).

2.1.2 Potential pitfalls

Strain. Multiple strains of inbred mice have been generated and maintained. The profound effects on mouse strains when creating a transgenic mouse must be taken into consideration. Knocking-out the same gene in two different mouse strains can lead to different phenotypes, since unlinked genes in the background strain can significantly affect the disease phenotype. The different mouse strains differ in, for example, their performance in the Morris water maze test, and in their visual and auditory abilities (Picciotto & Wickman, 1998).

Differences in the life-span. Certain human disorders with late onset, may be difficult to model in mice. For example, in the case of some lysosomal storage diseases, a disease process might take years to develop in humans. It is possible that the same process does not have time to develop within the mere two years of a mouse lifespan. However, this is still debated, and the alternative opinion claims that a human lifespan equals a mouse lifespan, regardless the difference in years. The importance of the absolute timescale may be good to keep in mind (Suzuki et al., 2003a).

Compensatory genes. Some gene knockout mouse models do not show any obvious phenotypes. It is possible that the absence of a given gene is compensated for by other gene(s) (Thyagarajan et al., 2003). A well known example for this is the Hexa knock-out mouse, which was designed to model Tay-Sachs disease. Yet the mouse proved to have no neurological symtoms, due to the fact that there is a significant difference in the GM2 ganglioside metabolism between mice and humans (Taniike et al., 1995; Sango et al., 1995).

Developmental state of birth. Mice and humans are not equally developed at birth. Using the brain as an example, human neuronal proliferation already occurs before birth. In mice this proliferation of neurons occurs postnatally, about 7-8 days after birth. The same differences apply to the active period of myelination, which occurs in mice approximately 20-25 days after birth, but takes place in the human brain right after birth. This is important when considering certain therapies. If a treatment is effective in a mouse only when given before the age of 10 days, then the same treatment should be administered to humans already *in utero*, in order to be effective (Suzuki et al., 2003a).

Environment. Mouse models for diseases are usually maintained in pathogen free facilities (SPF). This raises an interesting question as to whether a disease phenotype is dramatically affected by pathogens in non-infectious diseases, such as recessively inherited lysosomal storage diseases.

2.2 Lysosomes and lysosomal storage disorders

2.2.1 Lysosomes and lysosomal proteins

Lysosomes were discovered in 1955 by Christian de Duve, although the first lysosomal storage disease (LSD), Tay-Sachs's disease, was already described in 1881. De Duve and colleagues described lysosomes as a "new group of particles with lytic properties". Today we know that lysosomes are membrane-bound organelles with an acidic pH, containing vesicles with hydrolytic enzymes (Vellodi, 2005).

Lysosomes are primarily the "break-down stations" of the cell as it is in them that substrate breakdown occurs. Macromolecules that have to be degraded are delivered to the lysosomes by endocytosis, autophagy, and phagocytosis or by direct transport across the lysosomal membrane (Sleat et al., 2005). Lysosomes additionally have functions in, downregulation of surface receptors, release of endocytosed nutrients, inactivation of pathogenic organisms, repair of the plasma membrane and loading of processed antigens onto MHC class II molecules (Mullins & Bonifacino, 2001).
There are 50-60 hydrolytic enzymes and associated proteins contained within the lysosomes (Journet et al., 2002). They can degrade most macromolecules such as proteins, carbohydrates, lipids, and nucleic acids (Beck, 2007). The majority of the lysosome's enzymes are localized to the luminal compartment and are generally soluble glycoproteins. Products resulting from hydrolysis are subsequently translocated from the intralysosomal compartment across the membrane and released into the cytoplasm for reuse (Bagshaw et al., 2005).

Proteomic analyses have shown that lysosomes contain up to 215 integral membrane proteins, with different functions (Bagshaw et al., 2005). They maintain the acidic environment of the lysosome lumen, transport amino acids, fatty acids, carbohydrates and nutrients from the lysosome to the cytosol for reutilization and mediate fusion with other organelles (Beck, 2007; Eskelinen et al., 2003)

Lysosomal enzymes are synthetized in the endoplastic reticulum (ER) where they undergo several different modifications, such as removal of their signal sequence and N-glycosylation. From the ER they are further transported to the Golgi apparatus where a mannose-6-phosphate (M6-P) tag is attached to their sugars for targeting transport to the lysosomes (Kornfeld & Mellman, 1989) (Fig 1). However this step is not required for all lysosomal enzymes. Beta-glucocerebrosidase, a lysosomal membrane associated protein, does not require the M6-P ligand, but is instead targeted to the lysosomes by LIMP-2 (Reczek et al., 2007). Whilst some lysosomal enzymes, like Cathepsin D (Glickman & Kornfeld,

1993), sphingolipid activator proteins (SAPs) (Lefrancoiset al., 2003) and acid sphingomyelinase (Ni & Morales, 2007) use the sortillin receptor as an alternative route to the lysosomes.
From the trans-Golgi network (TGN) the M6-P receptor-protein complex moves on to late endosomes, where the M6-P receptor is cleaved. The lysosomal enzyme is then transported to the lysosomes, while the receptor is recycled back to the TGN. In the lysosomes the proteins may undergo further protein modifications, such as proteolysis, folding and oligomerization (Vellodi, 2005).

Lysosomal membrane proteins are not modified with M6P groups and therefore do not depend on the MPR for sorting. The targeting of lysosomal transmembrane proteins is instead mediated by short, linear sequences of amino acids, within the cytoplasmic domains of the proteins (Bonifacino & Traub, 2003). These signals include dileucine-based and tyrosine-based motifs and interact with clathrin coat components, to be transported to the lysosomes. The targeting of the lysosomal membrane proteins can additionally be regulated by lipid modifications, as has been shown for both Cln3 and mucolipin-1 proteins (Braulke & Bonifacino, 2008). Defects in any of the lysosomal proteins, in their targeting, activation and in their biogenesis, can all lead to lysosomal storage disease.

2.2.2 Lysosomal storage disorders

Today over 50 lysosomal storage diseases (LSDs) are known. They are characterized by intra-lysosomal accumulation of ungraded metabolites. LSDs are predominatly monogenic and resessively inherited, only Hunter's disease, Fabry's disease and Danon disease are not ingherited in this manner. LSDs have a tendency to be multisystemic and progressive. LSDs are due to defects in the breakdown of almost all types of molecules except for nucleic acids. Therefore, LSDs include lipidoses, mucopolysaccharidoses, oligosaccharidoses, and disorders of protein catabolism (Tardy et al., 2004). Several mutations in the same gene have been described for most of the LSDs. In some diseases the phenotypic variability can be explained by residual enzyme activity, however, no obvious genotype-phenotype correlations have been recognized. Patients with a similar genetic background or with the same disease mutation can present totally different phenotypes (Futerman & van Meer, 2004).

LSDs can be caused by (i) the deficiency of a given lysosomal hydrolase (the majority of LSDs), (ii) the deficiency of a protein that either activates or stabilizes one or several lysosomal hydrolases (as in GM2-activator deficiency, prosaposin deficiency, or galactosialidosis), (iii) the deficiency of an enzyme that participates in a) processing (e.g., as in mucosulfatidosis) b) targeting (as in mucolipidoses type II and III) of lysosomal enzymes and (iv) by defects of the lysosomal membrane (Beck, 2007; Tardy et al., 2004)(Fig 1).

LSDs can predominantly be divided into infantile, juvenile and adult forms. The infantile forms are the most severe, display brain pathology and result in death within the first years of life. The adult forms are much milder with slowly developing symptoms that usually attack the peripheral organs, rather than the brain. Juvenile forms are intermediate between the severe infantile and the milder adult forms.

The symptoms of LSDs can be divided into neurological and peripheral. Around two-thirds of patients with LSDs develop neurological symptoms, which include seizures, dementia and brainstem dysfunction. Peripheral symptoms include hepatosplenomegaly, injury to the heart and kidneys, abnormal bone formation, muscle atrophy and blindness (Futerman & van Meer, 2004). LSDs are all characterized by their intra-lysosomal accumulation of unmetabolized substrates, which are likely to be the primary cause of the disease. However, the cellular pathways which lead to the variety of symptoms, and cause severe tissue damage, remain largely elusive. Remarkably, adult neurodegenerative disorders such as Alzheimer, Parkinson and Huntington diseases share many of the secondary events leading to pathology in the LSDs. Neurodegeneration, inflammation and hypomyelination are often associated with brain pathology in LSDs, and are thus discussed more thoroughly in the following chapter of the thesis.

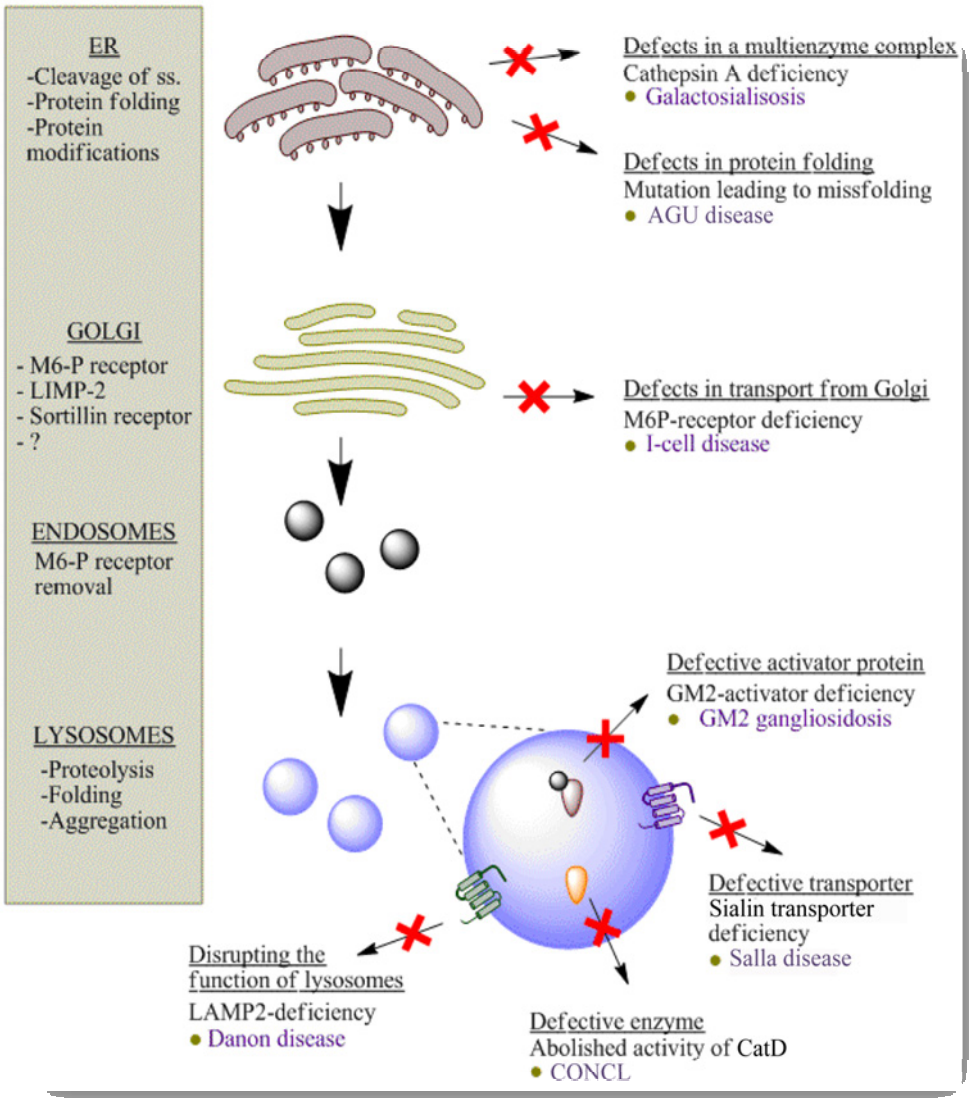

Figure 1. Cellular basis of Lysosomal storage diseases. ss.= signal sequence

2.2.3 Neuropathology of LSDs

LSDs are typified by production of an abnormal enzyme or other proteins of the endosomal lysosomal pathway, leading to accumulation of abnormally degraded or structurally aberrant molecules. The production of the abnormal enzyme/protein is the primary event that initiates a cascade of secondary events leading to pathology. In the central nervous system (CNS), these secondary elements include alterations in programmed cell death (PCD), as well as brain inflammation and demyelinization. These events lead eventually to neurodegeneration and affect the patients' cognition and behaviour. Neuronal loss, in LSDs, usually occurs at advanced stages of the disease. Many of these fetures occur also in adult neurodegenerative disorders. Thus, the improved knowledge of the events behind neurodegeneration can benefit not only children suffering from LSDs, but also adult patients suffering from more complex type of neurodegeneration.

2.2.3.1 Alterations in programmed cell death

Programmed cell death (PCD) plays a critical role in neural development and in normal physiological processes of the cell. When something goes awry, and the regulation of one or several pathways of PCD is disturbed, it can lead to neurodegeneration and disorders of the nervous system.
PCD is a complex phenomenon involving multiple pathways. It can be divided into at least three categories; ***apoptosis***, ***autophagic cell death*** and ***necrosis*** (Bredesen, et al., 2006). The type of cell death selected depends on the stimulus and the cellular context because every cell death program is a net result of self-propagating signals and variable factors that suppress the other cell death programs. Moreover, the different PCD mechanisms can operate in parallel or sequentially with each other, with multiple switchpoints between them (Boujrad et al., 2007) and different death mechanisms may operate in different parts of the same stressed neuron at the same time.

Apoptosis is a critical mechanism regulating neuronal cell number and proper connectivity in the developing nervous system. This makes it crucial for regulating brain development. Hallmarks of apoptosis have been observed both during neuronal development and neuronal cell death caused by acute and chronic injury (Yuan & Yankner, 2000). Apoptosis is characterised by distinct morphological features. They include cell shrinkage, chromatin condensation, blebbing of cytoplasmic membranes, and the fragmentation of cell bodies and nuclei into small

pieces that are called apoptotic bodies (Saraste, 1999). These apoptotic bodies are then phagosytosed by nearby macrophages or epithelial cells.

Apoptosis can be activated through three distinct pathways; the extrinsic and two intrinsic pathways. The extrinsic pathway originates from the cell surface through Fas receptors and tumour necrosis factor receptor 1(TNFR1). The major intrinsic pathway, or the mitochondrial pathway, originates from mitochondria and the less well known second intrinsic pathway originates from the endoplasmic reticulum (ER). The activation of the extrinsic pathway activates caspases 8 and 10 while both intrinsic pathways activate caspase 9. Caspases are proteolytic enzymes that cleave their substrates after specific aspartic acid residues. The extrinsic pathway is especially activated in pathological conditions in which inflammation is a prominent feature. The mitochondrial pathway is activated by stimuli such as heat, osmotic shock, DNA damage or growth factor starvation (Tardy et al., 2006). While stress in the ER, including the disruption of calcium homeostasis and accumulation of unfolded proteins in the ER (unfolded protein response, UPR), activate the second intrinsic pathway (Bredesen et al., 2006; Vila et al., 2003). Loss of control of apoptosis likely contributes to either degenerative (in case of too much cell death) or malignant (in case of too little cell death) diseases (Tardy et al., 2004).

Apoptotic cell death has been implicated as a cell death mechanism behind the pathology of common diseases like Alzheimer's and Huntington's disease (Ribe et al., 2008; Gil & Rego 2008). Impairment of lysosomal function may affect apoptosis in many ways and apoptosis has been detected in several LSDs, including several forms of NCLs. Accumulation of a toxic compound due to deficient degradation, lack of a molecule acting as an apoptosis suppressor, absence of proteolytic processing of a protein involved in apoptosis and abnormal apoptosis signalling due to perturbations in intracellular trafficking, are all possible mechanisms leading to neurodegeneration through apoptosis (Tardy et al., 2004). Among NCLs, there is evidence of apoptosis in mouse models for *Cln1, Cln3* and *Cathepsin D* deficiency (Gupta et al., 2001; Cotman et al., 2002; Koike et al., 2003; Seigel et al., 2002). In fact all of the above proteins; PPT1 (Cho, et al., 2000a; Cho & Dawson, 2000; Dawson et al., 2002), Cln3 (Puranam et al., 1999; Rylova et al., 2002;Persaud-Sawin et al., 2002) and Cathepsin D (reviewed in (Minarowska et al., 2007)) have themselves been implicated as playing a role in the regulation of apoptosis.

Autophagy is an evolutionarily conserved and strictly regulated lysosomal degradation pathway for cytoplasmic material and organelles. It maintains cellular turnower, and it is characterized by two-membrane autophagic vacuoles in the cytoplasm. It is induced under pathological conditions, such as amino acid starvation, hypoxia or intracellular accumulation of damaged organelles and cytoplasmic components. Its role is perceived to be the maintaince of free amino acids for use in various cellular functions. Depending on the delivery route, autophagy can be divided in macroautophagy, microautophagy, and chaperone-

mediated autophagy. Targets for degradation can for example be damaged mitochondria or misfolded protein aggregates. All autophagic vacuoles eventually fuse with lysosomes, which provide hydrolases as degrading enzymes (Klionsky & Emr., 2000; Bredesen, et al., 2006; Eskelinen & Saftig, 2008). In addition to its primary role in intracellular degradation, autophagy has also recently been shown to play an important role as an alternative cellular death mechanism. It has been reported to function in proliferation, death and differentiation during embryogenesis and postnatal development (Cecconi & Levine 2008).

In addition to the important housekeeping and quality control functions that contribute to health, autophagy has also been connected to the pathogenesis of several human diseases (Eskelinen & Saftig 2008), most likely due to errors in the regulation of it. It has been shown that knocking-out autophagy proteins in mice causes neurodegeneration and accumulation of ubiquitin positive protein aggregates (Hara et al., 2006; Komatsu et al., 2006). Furthermore, enhanced autophagy has been shown to reduce the toxicity of the protein aggregates in Huntington's disease, probably by preventing the formation of such aggregates (Ravikumar et al., 2004; Klionsky 2006). Defects in authophagy have also been implicated in several lysosomal storage diseases (LSDs). Most LSDs are caused by deficiencies of lysosomal hydrolases and therefore from accumulation of undegraded material in lysosomes. In these cases the authophagy-lysosomal fusion, and consequently degradation, is impaired, leading to neurodegeneraton. Such a mechanism has for example, been observed in mucopolysaccharidosis type IIIA (Settembre et al., 2008).

Increased autophagy has also been shown to be harmful. For example, in Nieman-Pick type C disease an increased autophagic process is considered to be the reason for death of Purkinje neurons (Ko et al., 2005). Autophagy is considered to be involved in the pathogenesis of NCL diseases, as is the case for the *Cathepsin D-/-* mouse (Koike et al., 2005). Autophagy has, furthermore, been shown to be activated in *Cln3* mice (Δex7/8) with the development of autophagic vacuoles being disrupted in these mice (Cao et al., 2006).

Necrosis is a form of PCD that includes swelling of the ER and mitochondria, and lacks typical apoptotic features such as apoptotic bodies and nuclear fragmentation (Bredesen et al., 2006). Necrosis was previously considered to be a passive form of cell death, but it has become clear that necrosis is controlled and can be activated by various stimuli, and certain developmental neuronal cell death has been found to exhibit features of necrosis. Necrosis has been shown to contribute to ovulation, immune defence, death of chondrocytes controlling the longitudinal growth of bones and cellular turnover in the intestine. Necrosis can lead to local inflammation,

presumably through the liberation of factors from dead cells that alert the innate immune system (Festjens et al., 2006).

Necrosis has also been connected with several neurodegenerative diseases, such as Alzheimer's disease, Huntington's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Although the molecular mechanisms of necrosis are not fully elucidated, it is thought that necrosis of adult neurons is mediated by increase of intracellular Ca^{2+}, mainly due to ER stress reactions, and that cytosolic calpains and spilled lysosomal cathepsins are the major players in necrotic neuronal death (reviewed in Artal-Sanz & Tavernrakis 2005). Very little is however, known about necrosis in LSDs. Necrosis has been suggested as a cell death mechanism in Niemann-Pick C disease (Erickson & Bernard 2002), but there are no reports to date, of necrosis in any of the NCLs.

Several pathological mechanisms exist that contribute to neurodegenerative diseases both in addition to/ or ultimately leading to PCD. These mechanisms can either act alone or in combination with each other. Suggested mechanisms include genetic factors, oxidative stress, excitotoxicity, protein aggregation, and damage to critical cellular processes, including axonal transport and organelles such as mitochondria (Shaw, 2005; Gil & Rego, 2008).

2.2.3.2 inflammation

The knowledge of the role of inflammation in the pathogenesis of neurodegenerative disease has increased rapidly in recent years. Neuroinflammation was previously considered to occur when damaged neurons induce an activation response from glia. Recent evidence, though, shows that glial cells (astrocytes, microglia, and oligodendrocytes) have "normal" housekeeping functions in the brain, which include transient upregulating of inflammation processes that are meant to be neuroprotective. These "normal" glial functions can then sometimes result in a more severe and chronic neuroinflammatory cycle that actually promotes neurodegenerative disease. Several possibilities exist for the relationship between inflammation and neurodegeneration: (1) that inflammation induces neurodegeneration; (2) that neurodegeneration causes inflammation; (3) other factors contribute to the development of inflammation and/or neurodegeneration; (4) inflammation and neurodegeneration participate in a cycle or a cascade in which they augment one another; and (5) that inflammation can protect against neurodegeneration. (Peterson & Fujinami, 2007)

Astrocytes. Astrocytes are the most abundant cells in the CNS, with the ratio of astrocytes to neurons appearing to rise with the increased complexity in the CNS.

Until relatively recently, astrocytes were believed to be structural cells, with a sole function of holding neurons together. It is currently known however, that astrocytes have several other functions. These functions include: amino acid-, nutrient-, and ion metabolism in the brain, coupling of neuronal activity and cerebral blood flow, and modulation of excitatory synaptic transmission (Maragakis & Rothstein, 2006). Astrocytes are known to release gliotransmitters, such as glutamate and D-serine. By releasing these gliotransmitters, in a process called *gliotransmission,* they can control neuronal synaptic activity. It has been suggested that defects in gliotransmission, either hyper- or hypoactivity, could contribute to disorders of the nervous system, such as epilepsy or schizophrenia (Halassa et al., 2007).

In response to CNS pathology, such as neurotrauma, brain ischemia, or neurodegenerative diseases, astrocytes can become reactive and migrate to the site of injury. This is known as *reactive gliosis* or *astrocytosis*. Reactive gliosis is characterized by a distinctive change in the appearance of astrocyte, called hypertrophy and by the proliferation of microglial cells and astrocytes. The best known hallmark for reactive gliosis is the upregulation of intermediate filament proteins, especially glial fibrillary acid protein (GFAP), but also vimentin and nestin (Pekny & Nilsson, 2005). This group of responses result in the formation of a tightly compacted limiting glial margin termed the astrogliotic scar, which is known to inhibit neurite outgrowth. These 'reactive' astrocytes may also aggravate tissue damage by releasing proinflammatory cytokines, such as tumor necrosis factor α (TNF-a), which can inhibit neurite outgrowth and kill oligodendrocytes. They can release, for example, nitric oxide (NO) and reactive oxygen species that can adversely affect cell survival after injury (reviewed in (Carmen et al., 2007; Liberto et al., 2004))

Although reactive gliosis has been considered the major obstacle to axonal regeneration, recent data suggest that in certain conditions, reactive astrocytes can actually support neurons. It has been shown that reactive astrocytes can metabolize the stored glycogen and in this way support neighboring neurons, by exporting glucose or lactate to them. Astrocytes can, furthermore, minimize damage and neuronal death by regulating harmful glutamate levels caused by excitotoxic neuronal death. They also mediate repair by secreting neurotrophic factors, such as nerve growth factor (NGF), NT-3, and glial cell line-derived neurotrophic factor (GDNF) and are known to promote remyelinisation and regulate neurogenesis during development. Astrocytes maintain the blood brain barrier (BBB) and participate in reforming it following CNS injury. They also participate in the synaptic structure by enveloping the synapse, and are in this way able to maintain synapses after injury and promote synaptogenesis (reviewed in (Carmen et al., 2007; Liberto et al., 2004)).

Microglia are considered to be the resident macrophages of the CNS, sharing many similarities with cells of the monocyte lineage (Ling & Wong, 1993). Microglia can become activated in response to, for example, infectious agents, damaged cells or tissues, altered molecules, or neurotransmitter imbalance (Schwartz et al., 2006). They may also be stimulated by astrocytes (McGeer & McGeer, 2008). Once activated, microglia undergo a dramatic transformation from their resting ramified state into an amoeboid morphology, they proliferate, upregulate major histocompatibility complex (MHC) molecules and secrete cytokines, chemokines, nitric oxide and reactive oxygen species. These cytotoxic substances are secreted in order to destroy infected neurons, viruses, and bacteria, but microglia can become overactive and cause large amounts of collateral damage by this mechanism. This could result in large scale neural damage, as the microglia destroy the brain in an attempt to abolish invading infections. Additionally, activated microglia can also become phagocytic, engulfing the offending material, such as bacteria, apoptotic cells, or myelin debris (Peterson & Fujinami, 2007; Block et al., 2007).

Traditionally it has been thought that the activated state of microglia can aggravate brain damage, and that decreasing microglial activation can be protective for the CNS. However, as is the case with astrocytes new data has emerged suggesting that microglia may also have neuroprotective functions. It would appear that the tilt toward harmful or beneficial outcomes is dependant upon the activating conditions (Hanisch & Kettenmann, 2007). It has been suggested that microglia may become dysfunctional as a result of aging, and would, therefore, be less equipped to sustain neuronal function. Microglia might also become dysfunctional from disease potentially aggravating the neurodegenerative process. Nonetheless, beneficial effects of microglia exist. Microglia are potentially promotors of migration, axonal growth, and terminal differentiation, of different neuronal subsets in the developing brain, conducted through the release of extracellular matrix components, soluble factors, and direct cell to cell contact (Vilhardt, 2005). In the adult CNS microglia are involved in processes such as tissue repair, neurotrophic support, induction of inflammation, or activation of lymphocytes.

On the whole, inflammation in the CNS can be described as a series of local immune responses that are recruited to damaged tissue, with the outcome ultimately dependent on its regulation. Therefore, whether inflammation is good or bad for recovery of the damaged CNS apparently depends on how it is regulated. Consequently, even if the presence of activated glia is observed in almost any neurological disease, they might have to be regarded as mainly beneficial, becoming destructive only when they escape from the strict control normally imposed on them (Schwartz et al., 2006).

Two disease states where microglial pathology has been shown to play a prominent role include Human immunodeficiency virus (HIV)(Garden, 2002) and the Prion diseases (v Eitzen et al., 1998). Microglia are also known to be affected in some rare genetic brain diseases, like PLO-SL (Kiialainen et al., 2005). Microglial pathology has not widely been studied in LSDs, but to date, it is known that widespread astrocytic activation is a general feature in the later stages of human and animal NCLs (Haltia, 2003). However, regionally defined reactive gliosis has recently been shown to be present in Cln3 (Pontikis et al., 2005), Cln5 (see 5.2.3 in this thesis) and Cln6 (Oswald et al., 2005; Kay et al., 2006), long before any signs of neurodegeneration or clinical symptoms become evident.

2.2.3.3 defective myelinisation

Oligodendrocytes are mature glial cells that myelinate axons in the brain and spinal cord. Myelin, a key component of brain white matter is formed from an extended, modified oligodendrocyte plasma membrane surrouding a portion of an axon in a spiral fashion. An oligodendrocyte may wrap up to 40 axons. Along one axon, neighboring myelin segments might belong to different oligodendrocytes (Baumann et al., 2001).

The brain is still immature at birth, with most of the process of myelination occuring within the two first years of life. The myelin membrane is a lipid bilayer composed of cholesterol, phospholipids and glycolipids, interrupted by proteins. The two major myelin proteins are; myelin basic protein (MBP) and proteolipid protein (PLP), which together represent 80% to 90% of the total myelin protein. Other proteins include myelin-associated glycoprotein (MAG), myelin-oligodendrocyte glycoprotein (MOG), and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP). Myelination enables fast conduction along the axon, and loss of myelin decreases the conduction velocity and destabilizes the molecular structure of the axonal cytoskeleton. Myelin is distributed in small segments along the axon. In the small regions between the segments, the nodes of Ranvier, clusters of sodium channels enable the axon membrane to produce action potentials (Lyon et al. 2006; van der Knaap et al., 2001). Due to these important functions of myelin, oligodendrocytes have important functions in maintaining axon integrity and neuron survival as well as providing support for neuronal somas, by synthezising several neurotrophic factors (McTigue & Tripathi 2008).

CNS white matter may be affected in different ways, with major differences in the underlying pathology. There can be a permanent myelin deficiency, the process of

myelination can be disturbed, the myelin sheath may be lost, myelin splitting may occur, extracellular oedema may involve CNS white matter, the white matter can be affected by gliosis, and lastly axonal damage can affect the myelin integrity. The situation is usually complex with the concurrence of several types of pathology (van der Knaap et al., 2001). White matter abnormalities are known to play a role in the pathogenesis of many neurological diseases in human. Several lysosomal storage disorders are included in this category. Diseases with myelin defects include; defects in genes encoding myelin proteins, peroxisomal diseases, mitochondrial diseases, disorders of amino acid and organic acid metabolism, defects of nuclear DNA repair, muscular dystrophies, macro/micro deletion syndromes, neurocutaneous syndromes and others (Lyon et al. 2006; van der Knaap et al., 2001). All white matter diseases share a similar set of clinical features. A period of normal development usually precedes the onset of neurological signs and symptoms. Then spasticity, motor weakness and ataxia develop, followed by optic atrophy and in some cases by seizures. Patients also suffer from behavioral and cognitive changes (Lyon et al. 2006).

Many myelin mutant mouse models have been analysed to clarify the relationships between myelin-, axon-, lipid- and immunopathology. These models have revealed different genetic defects of myelin (Baumann et al., 2001). Jimpy and Shiverer are two examples of mouse models with white matter defects due to mutations in myelin structural proteins, PLP (Knapp et al., 1986; Duncan et al., 1989) and MBP (Roach et al., 1983; Wiktorowicz et al., 1991) respectively. The absences of either of these two proteins results in oligodendrocyte death and myelin breakdown. The CNS of the shiverer mouse is hypomyelinated, but the peripheral nervous system (PNS) appears normal. The myelin of the CNS, wherever present, is not well compacted and lacks the major dense line (Readhead & Hood 1990).

Mutations involving enzymes of lipid metabolism can also induce myelin defects. The twitcher mouse, a model of Krabbe´s disease (belonging to the LSD disease group), suffers from mutations in the gene coding for galactosylceramidase (GALC)(Suzuki et al., 2003b). It is suggested that the accumulation of psychosine, a toxic metabolite, which is also a substrate for GALC, leads to apoptotic death of oligodendrocytes and subsequent demyelination (Jatana et al., 2002). Other mechanisms may also be involved in the processes of demyelination, and include; inflammation, oxidative stress, neurotransmitters (especially glutamate), heat shock proteins and matrix metalloproteinases (Baumann, et al., 2001). Loss of myelin is observed in several forms of NCLs (A. Haapanen, submitted). However, the mechanism(s) leading to these myelin defects are still unknown.

2.3 NCL diseases

Neuronal ceroid lipofuscinoses (NCLs) are a family of inherited pediatric neurodegenerative disorders, with an incidence of 1:12 500 in the Nordic countries and in the U.S.A. Elsewhere in the world the incidence is approximately ten times lower (Santavuori, 1988). NCLs are classified as lysosomal storage disorders (LSDs) since they present accumulation of membrane-bound intracellular protein aggregates (Futerman & van Meer, 2004; Kyttala et al., 2006), and are caused by mutations in endosomal-lysosomal proteins or proteins of the endoplasmic reticulum (ER) (Weimer et al. 2002). However, contrary to most other LSDs the storage material in NCLs is not a disease specific substrate (Elleder et al., 1993).

The NCLs were initially categorized into four different groups, based on age of onset; *infantile form* (INCL, age of onset 6-24 months, CLN1), *late- infantile form* (LINCL, age of onset 2-8 years, CLN2), *juvenile form* (JNCL, age of onset 4-10 years, CLN3) and *adult form* (ANCL, age of onset 11-50 years, CLN4). Later many different variants were discovered, which did not fit into the original categories, due to delayed age of onset or less severe, or protracted symptoms (Table 1)(Mole et al., 2005).

NCLs are autosomally recessively inherited, with the exception of some rare dominant adult forms (Peltonen et al., 2000). Currently, it is known that NCL can be caused by approximately 160 mutations in 8 known NCL-genes, CLN1, CLN2, CLN3, CLN5, CLN6, CLN7, CLN8 and CLN10 (http://www.ucl.ac.uk/ncl/)(Siintola et al., 2006a; Siintola et al., 2006b; Siintola et al., 2007; Steinfeld et al., 2006). The molecular genetic basis for CLN4 and CLN9 are still unknown. It is probable however, that the number of NCL genes is larger, since mutations in another lysosomal protein CLCN7, cause osteopetrosis and an NCL-like disease in mice (Jentsch, 2008). Existing family material implicates that at least three more LINCL-causing genes exist (A. Lehesjoki, unpublished).

The major hallmarks for NCLs are the death of selective neuronal populations and the progressive accumulation of autofluorscent ceroid-lipopigments. The clinical manifestations are generally similar in all forms including; visual impairment caused by retinal degeneration and eventually leading to blindness, sleep problems, motor abnormalities, epilepsia, dementia and finally premature death (Haltia et al. 2006; Haltia et al. 2003; Williams et al., 2006).

Although several of the gene defects for many of the NCL disorders have been known for many years, the pathology and the events leading to these disorders are still largely unknown.

Gene	Disease	Congenital	Infantile	Late Infantile	Juvenile	Adult
CLN1	INCL		O	O	O	O
CLN2	LINCL		O	O		
CLN3	JNCL				O	
CLN4	ANCL					O
CLN5	$vLINCL_{Fin}$			O	O	
CLN6	vLINCL			O		
CLN7	tLINCL			O		
CLN8	EPMR/NE			O	O	
CLN9	vJNCL				O	
CLN10	CONCL	O		O		

Table 1. Ages of onset for different forms of NCL

2.3.1 Congenital and Infantile NCLs

2.3.1.1 Congenital NCL/ CLN10

The congenital neuronal ceroid lipofuscinosis (CONCL) is the earliest and most aggressive form of NCLs. This disorder is very rare and only a few cases have been described. The patients suffer from microcephaly, respiratory problems, rigidity and epileptic attacks, dying within hours to weeks after birth. A massive loss of cortical neurons as well as extensive gliosis and absence of myelin can be seen in the brains of the patients (Steinfeld et al., 2006). Autofluorescent storage bodies, typical for NCLs, with a granular osmiophilic deposit (GROD) ultrastructure are observed in the patient cells. The GRODs reside in the lysosomes and resemble autofluorescent lipofuscin pigments that accumulate in cells during normal aging. However, GRODs rarely contain lipid droplets, which differentiates them from normal aging pigments (Lu,et al. 1996; Tyynelä, et al. 1993). GRODs have a packed globular structure which is more granular and tightly packed in neurons and coarser and looser in non-neuronal cells. GRODs can be detected for diagnostic purposes in blood lymphocytes and skin dermal cell types (Mole, et al. 2005). The major storage material in CONCL consists of sphingolipid activator protein D (Saposin D). However, the storage material seems to vary between species, accumulation of saposins A and D (similarly to INCL) can be observed in humans and sheep

(Tyynela et al., 2000) while subunit c of the mitochondrial ATPase (similarly to other NCLs, except for INCL) is found in mice (Koike et al., 2000).

Mutations in Cathepsin D (*CTSD*), located on chromosome 11p15.5, (Faust et al., 1985) were quite recently discovered to be the cause for this disease (Siintola et al., 2006b; Steinfeld et al., 2006). *CTSD* is an aspartic protease that localizes to the lysosomes (Tang & Wong, 1987; Dittmer et al., 1999). Several *in vitro* substrates have been assigned to *CTSD*, including prosaposin (*proSAP*), which can be cleaved into saposins A and D (Gopalakrishnan et al., 2004), but no *in vivo* substrates are yet known. *CTSD* has, however, been implicated to function in processes related to cell proliferation, antigen processing, apoptosis, and regulation of plasma HDL-cholesterol levels (Table 2)(Berchem et al., 2002; Moss et al., 2005; Haidar et al., 2006; Benes et al.,2008).

One patient with a mutation in *CTSD* is known to present a disease course resembling more variant late infantile forms of NCLs than CONCL (Steinfeld et al., 2006; See 2.3.2.6 in this thesis).

2.3.1.2 Infantile NCL /CLN1

The infantile neuronal ceroid lipofuscinosis (INCL) will be discussed in chapter 2.3.6.

2.3.1.3 Other NCLs with infantile onset

Some patients with mutations in the *CLN2* gene (see 2.3.2.1), that usually manifest in a late infantile disease, are known to have an infantile onset disease (Ju et al., 2002).

2.3.2 Late infantile and variant NCLs

2.3.2.1 Late infantile NCL/ CLN2

The late infantile neuronal ceroid lipofuscinosis (LINCL) manifests in late infancy, between the ages of 2-4. The *CLN2* gene deficient in the disease is located on chromosome 11p15 and codes for a pepstatin insensitive carboxyl protease called tripeptidyl peptidase I (TPP I) (Sleat et al., 1997). TPP I is a lysosomal serine-carboxyl proteinase that removes tripeptides from the N-termini of polypeptides (Table 2.)(Sleat et al., 1997; Golabek & Kida, 2006). 56 mutations in the *CLN2* gene

are known to date (http://www.ucl.ac.uk/ncl/), but no genotype-phenotype correlation has been reported.

The ultrastructural feature of the storage material in LINCL patients is curvilinear profiles that aggregate in membrane-bound lysosomal residual bodies. The curvilinear body is a hallmark for LINCL mutations and it is a reliable diagnostic target. It can be observed in within the nervous system and extraneural tissues, including blood lymhocytes (Mole et al, 2005). The major storage component in LINCL is subunit c of the mitochondrial ATPase. Patients suffer from widespread neuronal loss in their brain, manifesting especially in the cerebellum and hippocampus (Palmer et al., 1992).

No *in vivo* substrates are known for TPP I, but it is known that TPP I can cleave subunit c *in vitro* (Ezaki et al., 1999). TPP I reportedly interacts with two other NCL proteins, namely CLN3 and CLN5, based on coimmunoprecipitation and in vitro binding assays, but the relevance of these interactions and the *in vivo* function of TPP I itself remains elusive (Vesa et al., 2002).

In addition to classical LINCL, mutations in the *CLN2* gene are also known to cause juvenile- (Hartikainen et al., 1999; Sleat et al., 1999; Steinfeld et al., 2002; Wisniewski et al., 1999) and infantile-forms (Ju et al., 2002) on NCL.

2.3.2.2 The Finnish variant late infantile NCL / CLN5

The Finnish variant form of late infantile neuronal ceroid lipofuscinosis ($vLINCL_{Fin}$) is a variant form of LINCL mostly affecting Finnish patients. $vLINCL_{Fin}$ will be discussed in chapter 2.3.5.

2.3.2.3 Variant late infantile NCL/ CLN6

Variant late infantile neuronal ceroid lipofuscinosis (vLINCL), resembles phenotypically the classical LINCL, with a similar disease phenotype, apart from a later onset (3-8 years) and a slower progression (Mole et al., 2005). vLINCL results from mutations in the *CLN6* gene, positioned on chromosome 15q23 (Sharp et al., 1997). Presently 27 disease mutations are known, with little variation in their disease phenotype (http://www.ucl.ac.uk/ncl/).

The *CLN6* gene codes for a transmembrane protein with 7 membrane-spanning domains. The CLN6 protein is localized in the endoplasmic reticulum (ER). No function has yet been assigned for CLN6, although the defect of this protein results in lysosomal dysfunction (Table 2) (Heine et al., 2004; Mole et al., 2004). Lamina V of the cortex is most severely affected by neuronal loss in vLINCL patients (Elleder et al., 1997).

As in other variant LINCLs, the major storage component in vLINCL is subunit c of the mitochondrial ATPase, and the ultrastructure of the storage material show fingerprint-, curvilinear- and rectilinear profiles (Elleder et al., 1997). Fingerprint profiles are membrane-bound, electron-dense bodies composed of paired parallel dark lines separated from each other by a lighter intervening layer of varying thickness. They may be mixed with curvilinear or rectilinear profiles even within the same storage cytosome (Haltia 2003).

2.3.2.4 The Turkish variant late infantile NCL /CLN7

The Turkish variant form of late infantile neuronal ceroid lipofuscinosis (tLINCL) is caused by a defect in the newest member of the NCL gene family, the *MFSD8* gene located on chromosome 8q28.1-q28.2 (Siintola et al., 2007). The clinical course in tLINCL is similar to that of classical LINCL, apart from having more severe seizures. The age of onset ranges between 2 and 7 years of age (Mitchell et al., 2001; Topcu et al., 2004).

The ultrastructure of the storage material consists of fingerprint-, curvilinear- and/or rectilinear profiles and the major storage component is subunit c of the mitochondrial ATPase (Mitchell et al., 2001; Topcu et al., 2004).

Six mutations have been identified in the *MFSD8* gene (http://www.ucl.ac.uk/ncl/), which codes for a polytropic integral membrane protein with 12 transmembrane domains (Siintola et al., 2007). The lysosomal MFSD8 protein is suggested to be a member of the major facilitator superfamily (MFS), which is a large protein family of secondary active transporters. This family of proteins is known to carry small soluble compounds, such as sugars, drugs, inorganic as well as organic cations and many metabolites (Kasho et al. 2006). Neither substrate specificity nor functions of the MFSD8 protein are known (Table 2).

2.3.2.5 variant late infantile NCL / CLN8

The *CLN8* gene, which is localized on chromosome 8p23, codes for the CLN8 protein (Ranta et al., 1999; Lonka, et al. 2000). CLN8 is a membrane protein with, probably four-seven transmemrane domains. CLN8 is known to cycle between the ER and the ER-Golgi intermediate compartment (ERGIC) (Lonka, et al. 2000), and belongs to the Tram-Lag1p-CLN8 (TLC) protein family. Even though the function of CLN8 is unknown, TLC-proteins are known to participate in the biosynthesis, metabolism, transport and sensing of lipids (Table 2)(Winter & Ponting, 2002). Recently TLC-proteins have been linked to the regulation of acyl-CoA dependent ceramide synthesis (Riebeling et al., 2003).

Mutations in the CLN8 protein gives rise to two phenotypically different NCL disorders; Progressive epilepsy with mental retardation (EPMR) or Northern epilepsy and vLINCL (Ranta et al., 1999; Ranta et al., 2000; Cannelli et al., 2006; Zelnik et al., 2007). The more severe vLINCL is discussed here, but as Northern epilepsy is the mildest form of childhood NCLs it will be discussed with the other Juvenile NCLs in chapter 2.3.3.2.

vLINCL patients were initially thought to suffer from tLINCL, before mutations in the *CLN8* gene were discovered. Nonetheless, the clinical symptoms resemble those of the other variant LINCLs, with an age of onset between 2-7 years of age (Topcu et al., 2004). To date 11 mutations in the *CLN8* gene are found to result in disease (http://www.ucl.ac.uk/ncl/). The storage material of the patients consists of subunit c of the mitochondrial ATPase and shows fingerprint- and/or curvilinear profiles as well as occasional GRODs (Topcu et al., 2004; Ranta et al., 2004; Cannelli et al., 2006; Zelnik et al., 2007).

2.3.2.6 Other NCLs with late infantile onset

A small group of both CONCL (Steinfeld et al., 2006) and INCL (Das et al., 1998) patients, usually suffering from disorders of early infancy have disease courses that resemble that of LINCL. Additionally, existing family material of variant late infantile NCLs suggests that at least three more genes causing LINCL are still to be identified (A Lehesjoki, unpublished).

2.3.3 Juvenile NCLs

2.3.3.1 Juvenile NCL / CLN3

The juvenile form of neuronal ceroid lipofuscinosis (JNCL) is also known as Batten disease and is globally the most common form of NCLs. The *CLN3* gene defective for JNCL located on chromosome 16p12 encodes for a protein of the same name. The CLN3 protein is a hydrophobic integral membrane protein with, most likely, six transmembrane domains (Consortium. 1995; Kyttälä et al., 2004; Nugent et al., 2008). CLN3 localizes predominantly to the lysosomes (Jarvela et al., 1998). The neuronal localisation differs slightly from extra neural cells, because CLN3 is additionally reported to localize to early endosomes and synaptosomes (Kyttala et al. 2004; Storch et al., 2007; Luiro et al., 2001). Small amounts of CLN3 have also been shown to localize to the plasma membrane, in lipid rafts in neurons (Rakheja et al., 2004). More than 40 mutations in *CLN3* are known to result in JNCL

(http://www.ucl.ac.uk/ncl/). Most of these result in juvenile onset disease, but atypical protracted or delayed disease forms are also known to exist. The genotype-phenotype correlation is somewhat unclear, but recent evidence suggests that mutations in distinct domains of CLN3 result in different phenotypes, indicating several functional roles for CLN3 (Kitzmuller et al., 2008).

The JNCL disease begins with visual failure, somewhat later than in the late infantile forms, between the ages of 4-10 years. In addition to the more typical NCL symptoms, JNCL patients also suffer from different psychiatric symptoms, such as aggressiveness, depression and sleep problems (Jarvela et al., 1997; Santavuori et al., 1993; Santavuori et al., 2000; Williams et al., 2006).

As in most other NCLs, subunit c of the mitochondrial ATPase is the major storage material and the ultrastructure consists of fingerprint profiles (Carpenter et al., 1977).

Many different functions have been assigned to CLN3. It is thought to participate in the maintenance of lysosomal pH, in arginine transport and in the modification of proteolipids. It is also suggested to participate in some of the cell death mechanisms, in autophagy and in the prevention of apoptosis. CLN3 might also take part in cytoskeleton linked processes and in both vesicular transport as well as with membrane trafficking (Table 2) (reviewed by (Kyttala et al., 2006). Recent results have also linked CLN3 to fodrin-mediated endocytosis of Na^+,K^+,ATPase (Uusi-Rauva et al., 2008).

Related to the mentioned functions, CLN3 is reported to interact with Hook1, based on in-vitro binding (Luiro et al., 2004), Calsenilin, based on in-vitro binding and immunoprecipitation assays (Chang et al. 2007), Na^+, K^+ ATPase and Fodrin, based on yeast two-hybrid screening (Uusi-Rauva et al., 2008). Furthermore, CLN3 interacts with two other NCL proteins, CLN2 and CLN5, based on coimmunoprecipitation and in vitro binding assays (Vesa et al., 2002).

2.3.3.2 Northern epilepsy/ CLN8

Northern epilepsy is the mildest form of childhood NCLs and manifests exclusively in Finnish patients. The age of onset is between 5-10 years of age and it is caused by mutations in the *CLN8* gene (Ranta & Lehesjoki, 2000). Mutations in *CLN8* are known to produce a more severe NCL, vLINCL, which is discussed in chapter 2.3.2.5.

All, but one, Finnish Northern epilepsy patients are homozygous for the same missense mutation (Ranta et al., 1999). The patients suffer from generalized tonic-clonic seizures. In contrast to all other childhood NCLs, none of the patients suffer

from retinal degeneration (Ranta & Lehesjoki, 2000). Progressive cerebellar and brainstem atrophy is evident already in young adults (Lang et al., 1997).

The storage material consists of subunit c of mitochondrial ATPase and the ultrastructure is curvilinear as well as granular (Hermansson et al., 2005).

2.3.3.3 CLN9

The CLN9 disease is the first of the two NCLs left with undiscovered gene defects. CLN9 resembles JNCL to a degree, but can be classified as its own entity based on the differing gene expression pattern and the phenotype of CLN9 patient cells. The characteristics for these fibroblasts include; rapid growth, sensitivity to apoptosis, defects in cell adhesion and reduced levels of ceramide, dihydroceramide as well as sphingomyelin (Schulz et al., 2004).

As mentioned previously no gene defects have been found to date and all other known NCL genes have been excluded as candidates. Nevertheless, CLN8 has been shown to partially correct the phenotype of *CLN9*-/- cells (Guillas et al., 2001). Based on the data collected from CLN9 patient cells there has been suggestions that the CLN9 protein might function in regulating dihydroceramide synthase (Table 2)(Schulz et al., 2006).

The ultrastructure of the storage material comprises of GRODs, fingerprint and curvilinear profiles. Subunit c of the mitochondrial ATPase has been found in one patient (Schulz et al., 2004).

2.3.3.4 Other NCLs with juvenile onset

Mutations in the *CLN1* gene, normally resulting in INCL, are known to sporadically cause a juvenile onset disease (Mitchison et al., 1998), whilst a mutation in the *CLN5* gene, normally resulting in $vLINCL_{Fin}$ is known to result in a juvenile onset disease in one Colombian family (Pineda-Trujillo et al., 2005).

2.3.4 Adult NCLs

2.3.4.1 Adult NCL or CLN4

The second form of NCLs with a yet undiscovered gene defect, that has been given the genetic assignment CLN4, is the adult neuronal ceroid lipofuscinosis (ANCL). ANCL is the mildest form of NCLs with an age of onset ranging from 11 up to 50 years of age (Berkovic et al. 1988). The gene defect is thought to be most likely

heterogeneous and other NCL genes are regarded as possible candidates. As an example, mutations in the *CLN1* gene (INCL) can produce an adult-onset NCL (van Diggelen et al., 2001).

Other features that support the hypothesis of a heterogeneous genetic background, include; the finding of the ultrastructure of the storage material which varies between GRODs, fingerprint, curvilinear and rectilinear profiles and the storage material containing both subunit c of the mitochondrial ATPase and Saposins A and D (Berkovic et al., 1988).

In most cases ANCL is inherited in an autosomal recessive manner, and referred to as Kufs disease. However, in some cases the mode of inheritance is autosomal dominant and in these cases ANCL is referred to as Parry disease (Berkovic et al., 1988).

2.3.4.2 Other NCLs with adult onset

Three adult cases have been described with mutations in the *CLN1* gene, normally resulting in an infantile onset disease. The disease onset occurs in the third or fourth decade of life and the disease progress is slow (van Diggelen et al., 2001; Ramadanet al., 2007).

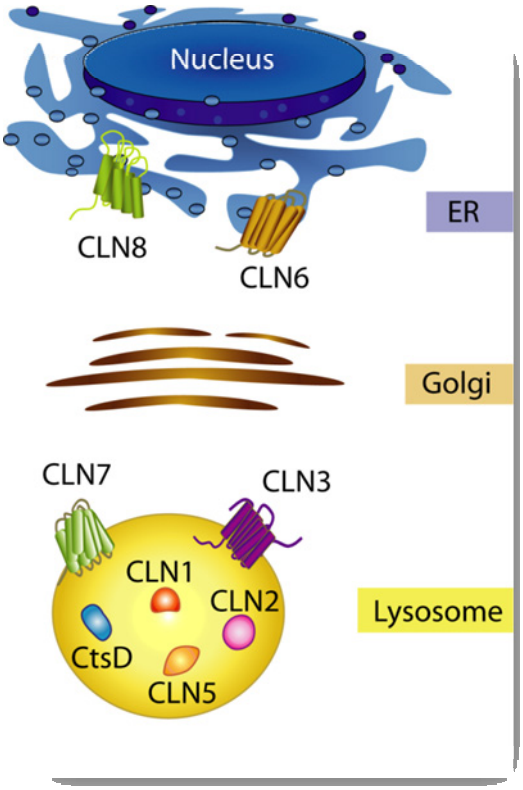

Figure 2. The cellular localization of the known NCL proteins. CLN6 and CLN8 are localized in the endoplasmic reticulum (ER), whereas CLN1/PPT1, CLN2/TPP1, CLN3, CLN5, CLN7 and CLN10/Cathepsin D (CtsD) are localized in the lysosomes. CLN3, CLN6; CLN7 and CLN8 are transmembrane proteins, while CLN1,CLN2, CLN5 and CtsD are soluble.

2.3.5 CLN5

2.3.5.1 Clinical features

The Finnish variant late infantile neuronal ceroid lipofuscinosis ($vLINCL_{Fin}$) was originally described in Finnish patients. $vLINCL_{Fin}$, represents a typical example of a disease belonging to the Finnish disease inheritage (Santavuori, et al., 1982). $vLINCL_{Fin}$ resembles the classical LINCL, apart from the later onset and slower course of the disease. The first symptoms of the disease appear usually between the ages of 4-7 years. They begin with attention deficits followed by motor clumsiness, learning problems and progressive visual failure, which eventually leads to blindness. Other typical symptoms include motor and mental detoriation, myoclonia, epileptic seizures and sleep disturbances. By the age of 10 years most patients are blind and they have lost both their ability to speak and to walk. $vLINCL_{Fin}$ patients die usually around their second decade of life, although variations occurs between 14-39 years (Santavuori et al., 1982; Santavuori et al., 1991). Mutations in *CLN5* can in some cases result also in a juvenile onset disease (Pineda-Trujillo et al., 2005).

The brain pathology of the patients reveals severe neuron loss both in the cortex and the cerebellum. The Purkinje and granular represent the major cell populations that are dying in the cerebellum (Santavuori et al., 1982), whereas neurons in laminae III and V are most vulnerable in the cortex (Tyynela et al., 1997). Reactive gliosis accompanies the neuron loss in the cortex (Santavuori et al., 1982). Progressive optical atrophy and loss of myelin add to the brain pathology of $vLINCL_{Fin}$ patients (Autti et al., 1997).

At the time of diagnosis, neuroimaging usually show generalized brain atrophy, with the cerebellum being predominantly affected. The periventricular white matter shows abnormally high signal intensity and the thalami show decreased signal intensity to the basal ganglia on T2-weighted images (Santavuori et al., 2000). Magnetoencephaography (MEG) of CLN5 patients has shown a selective enhancement of the early cortical response to median nerve stimulation (Lauronen et al., 2002). This may reflect defective interneuronal inhibition in the cortex.
The ultrastructure of the storage material resembles that of the other late variant NCL's, with rectilinear, curvilinear and fingerprint profiles. The main protein of the storage material is subunit c of the mitochondrial ATPase, although small amounts of Saposins A and D have also been found (Santavuori et al., 1982; Tyynela et al., 1997)

2.3.5.2 The CLN5 gene and protein

The *CLN5* gene was originally localized to chromosome 13q22by Savukoski and colleagues (Savukoski et al., 1998). The gene contains four exons and translates into a 407 amino acid protein with a molecular mass of 38-60 kDa, depending on glycosylaton (Isosomppi et al., 2002). 13 mutations in the *CLN5* gene have been found to result in $vLINCL_{Fin}$ (http://www.ucl.ac.uk/ncl/). 94% of the Finnish patients carry the major mutation, the $CLN5_{FinMajor}$, which results in a truncated protein, unable to be transported further than to the Golgi complex (Holmberg et al., 2000). The *CLN5* gene is conserved in eukaryotes and the protein has no known homologues, which has made it rather difficult to predict any functions for the protein. Consequently, the function(s) of CLN5 has remained elusive.

Human and mouse *CLN5/Cln5* are widely expressed in all tissues. Expression begins at an early developmental stage and increases during corticogenesis (Heinonen et al., 2000b; Holmberg et al., 2004). CLN5 is most likely to be a soluble lysosomal protein, with eight potential N-glycosylation sites. It is found in the M6-P proteome, suggesting that it is targeted to the lysosomes via the M6-P receptor pathway. Our recent results suggest, however, that *CLN5/Cln5* might also utilize an alternative pathway, due to the fact that it is present in the lysosomes in M6-P receptor deficient cells (Schmiedt & Bessa, manuscript in preparation). Additional locations, apart from the lysosomes, have been suggested for the CLN5 protein in neurons. It has been reported to localize to the cell soma as well as to neural extensions (Holmberg et al., 2004). Yet, no functions related to lysosomes or these alternative locations have been assigned for CLN5 (Table 2).

CLN5 interacts with CLN2 and CLN3, based on coimmunoprecipitation and in vitro binding assays (Vesa et al., 2002). These were the first interactions reported between any NCL proteins, and even though no functional relevance of these interactions has been found to date, it still implied, for the first time, that all NCL proteins could be connected.

2.3.6 CLN1

Infantile neuronal ceroid lipofuscinosis (INCL) is enriched in the Finnish population with an incidence of 1:20 000 (Santavuori et al., 2000). The INCL patients are symptom free at birth but start to manifest symptoms around 6 months of age. Delayed head growth (microcephaly) and muscular hypotonia are the only early signs of this devastating disease. The symptoms continue with visual failure, epileptic seizures, mental and motor deterioration, irritability and sleep problems.

Most patients have lost all their motor abilities and their vision by the age of three years. By this stage they have furthermore lost their social interest and suffer from increased spasticity, seizures and myoclonia. INCL patients usually die around 10 years of age (Santavuori et al., 1973; Santavuori et al 1974; Santavuori et al., 2000). Around 50% of all INCL patients suffer from this infantile form of NCL, but mutations in the CLN1 gene, can also result in late infantile (Das et al., 1998), juvenile (Mitchison et al., 1998) and even in some cases, adult onset variants of the disease (van Diggelen et al., 2001; Ramadan et al., 2007). It has been suggested that mutations near the active site of the protein result in an infantile form, whereas missense mutations in the peripheral parts of the protein result in later onset disease (Bellizzi et al., 2000; Das et al., 2001).

The brain pathology of INCL patients implicates a progressive neuron loss, reactive gliosis and macrophage infiltration in the cortex. The cortical neurons are almost totally lost by the age of three years. At that time, magnetic resonance imaging shows extreme cerebral and cerebellar atrophy with very high signal intensity in the white matter. The electroencephalography (EEG) becomes flat and the electroretinogram (ERG) is extinguished (Santavuori et al., 1999). The brain atrophy is striking when the brain is autopsied, revealing almost complete loss of both cerebral and cerebellar cortices. Adding to the severe brain pathology, an almost complete loss of myelin and atsrogliosis can be seen throughout the whole brain (Haltia et al., 1973).

INCL together with CONCL differ from the other NCLs concerning the ultrastructures of the storage materials, which comprise of GRODs. The major proteins in these inclusions have been determined to be saposins A and D (Tyynela et al., 1993) instead of subunit C, found in most other forms of NCL.

2.3.6.1 The CLN1 gene and protein

The gene defected in INCL is called *CLN1*. It was mapped to chromosome 1p32 by Vesa and colleagues in 1995 (Vesa et al., 1995). The gene consists of 9 exons and codes for a protein called Palmitoyl protein thioesterase or PPT1. 45 mutations in the *CLN1* gene are known to date (http://www.ucl.ac.uk/ncl/). The PPT1 protein consists of 306 amino acids and migrates as a 37/35 kDa doublet. It contains three N-linked glycosylation sites, which are required for both the stability and acticity of the protein. PPT1 has been crystallized, and it resembles a classical α/ß-serine hydrolase, a typical structure for lipases (Verkruyse & Hofmann, 1996; Bellizzi et al., 2000). PPT1 is expressed in most tissues and this expression is developmentally regulated. The expression of PPT1 appears to start early; the expression has been

detected as early as embryonic day 8 in mouse embryos (Isosomppi et al., 1999; Suopanki et al., 1999).

PPT1 is a soluble lysosomal enzyme. It is M6-phosphorylated and consequently uses the M6-P route to lysosomes (Verkruyse & Hofmann, 1996; Hellsten et al., 1996). However, many other locations have also been assigned to PPT1 in neurons. These include the cell soma, axonal varicosities, presynaptic terminals and especially synaptosomes and synaptic vesicles. (Ahtiainen et al., 2003; Heinonen et al., 2000a; Isosomppi et al., 1999; Lehtovirta et al., 2001; Suopanki et al., 2002). Additionally, PPT1 has been reported in lipid raft preparations (Goswami et al., 2005). The pH range for PPT1 activity is broad and unusually high for a lysosomal protein, which could imply functionality also outside of lysosomes.

2.3.6.2 Functions of PPT1

PPT1 is an enzyme that removes palmitate groups from other proteins. PPT1 is known to cleave palmitate from s-acylated proteins (Camp & Hofmann, 1993). It can depalmitoylate several proteins *in vitro*, such as H-Ras, Gap-43 and rhodopsin (Camp & Hofmann, 1993; Cho et al. 2000b). However, no *in vivo* substrates are known yet. Only another depalmitoylating enzyme is known apart from PPT1, acyl protein thioesterase or APT1 (Lu & Hofmann, 2006). Palmitoylation is a reversible protein modification that is attached to proteins post-translationally. Palmitoylation increases protein hydrophobicity, facilitates protein interactions with lipid bilayers, and can markedly alter protein sorting and function. It is known to modify numerous classes of neuronal proteins, including neurotransmitter receptors, synaptic scaffolding proteins and secreted signalling molecules (el-Husseini & Bredt, 2002). The exact physiological function of PPT1 is, however, not yet known. PPT1 has been proposed to participate in several different processes of the cell, such as apoptosis, endocytosis, vesicular trafficking, synaptic function and lipid metabolism (Table 2) (reviewed in (Jalanko & Braulke 2008).

PPT deficient lymphoblasts are known to be sensitive for apoptosis and overexpression of PPT1 appears to protect against apoptosis (Cho & Dawson 2000). Conversly, other results show that reduced expression of PPT1 increases the susceptibility for induced apoptosis. PPT1 deficient fibroblasts show an elevation of lysosomal pH, which might imply defects in endocytosis. Recent data shows that PPT1 interacts with the ectopic F1-ATP-synthase, based on surface plasmon resonance and GST-pulldown assays, which can function as an ApoA1 receptor at the plasma membrane (Kim et al., 2004; Martinez et al., 2003; Moser et al., 2001). The complete functional consequence of this interaction is yet unknown, but functional data has revealed that the plasma phospholipid protein (PLTP),

cholesterol and ApoA-1 levels are significantly reduced in the sera of Ppt1-deficient mice, which suggest a role for PPT1 in cellular lipid metabolism (Lyly et al., 2008).

Gene	Protein	Function	Interaction partners
CLN1	PPT1	Cleaves palmitate from other proteins, in apoptosis, endocytosis, vesicular trafficking, synaptic function and lipid metabolism.	F1-ATP synthase
CLN2	TPP1	A serine-carboxyl proteinase that removes tripeptides from the N-termini of proteins.	CLN3, CLN5
CLN3	CLN3	Functions in maintenance of lysosomal pH, arginine transport, modification of proteolipids, autophagy, prevention of apoptosis, cytoskeleton linked processes, vesicular transport, membrane trafficking and endocytosis of Na+,K+,ATPase	CLN2, CLN5, Hook1, Calsenilin, Na+, K+ ATPase, Fodrin
CLN4	-	-	-
CLN5	CLN5	-	CLN2, CLN3
CLN6	CLN6	-	-
CLN7	MFSD8	A suggested member of the major facilitator superfamily (MFS)	-
CLN8	CLN8	Belongs to the Tram-Lag1p-CLN8 (TLC) protein family	-
CLN9	-	Regulates the dihydroceramide synthase	-
CLN10	Cathepsin D	Functions in cell proliferation, antigen processing, apoptosis and regulation of plasma HDL-cholesterol levels	-

Table 2. The functions and interaction partners of NCL proteins

2.3.7 Mouse models of NCLs

Mouse models have proved to be invaluable tools for dissecting the disease mechanisms in NCLs. Mouse models are available for all but one of the genetically characterized NCL subtypes (Table 3). tLINCL caused by mutations in the recently characterized *MFSD8/CLN7* gene (Siintola et al., 2007) is not represented by a mouse model to date. All mouse models display phenotypes that resemble those of their respective human disorders (Table 3) (Cooper et al., 2006; Jalanko and Braulke et al., 2008).

2.3.7.1 Cln1

Two mouse models for *CLN1* have been produced by different targeting strategies; The *Cln1-/-* mouse made by Gupta and colleagues, has an insertion mutation in exon 9 (Gupta et al., 2001) and the Ppt1$^{\Delta ex4}$ mouse made by Jalanko and colleagues with a deletion in exon 4 (Jalanko et al., 2005). The mice display a severe phenotype, with a widespread inflammation-mediated neurodegeneration, resembling that of INCL, in the later stages of the disease.

Localized reactive gliosis as well as neurodegeneration is evident in the *Cln1* deficient mouse-models. These events are predominantly localized to the thalamo-cortical systems, with the earliest events described in the thalamus, especially thalamic relay nuclei of the visual system and later moving to the corresponding target neurons in the cortex, most likely as a consequence from the reduced signalling from the thalamus (Bible et al., 2004; Gupta et al., 2001; Kielar et al., 2007).

Gene expression studies in young *Cln1-/-* mice have revealed expression changes in immediate early genes, a group of genes that are defined as the first to be expressed following specific extracellular signals (Qiao et al., 2007). Inflammation related changes already dominate in 6 month old mice (Jalanko et al., 2005), whereas in 10 week old presymptomatic *Cln1-/-* mouse brains, changes in single genes were reported with no specific pathways reported (Elshatory et al., 2003). Expression profiling made on cultured neurons derived from Ppt1$^{\Delta ex4}$ mice revealed alterations in cholesterol metabolism, neuronal maturation and calcium homeostasis (Ahtiainen et al., 2007).

Studies on the *Cln1* deficient mice have revealed that the pool of readily releasable synaptic vesicles is diminished in *Cln1-/-* neurons (Virmani et al., 2005; Kim et al., 2008). PPT1 deficiency is known to cause persistent membrane sorting and retention of several synaptic vesicle (SV) proteins which are known to undergo palmitoylation. This is likely to disrupt the recycling and regeneration of fresh vesicles, thus disrupting neurotransmission (Kim et al., 2008). However, data from neuron cultures and acute brain slices reveal no alterations in the synaptic transmission in Ppt1$^{\Delta ex4}$mice (Ahtiainen et al., 2007). Furthermore, a deficiency linked to ER-mediated cellular stress leading to increased apoptosis is evident in *Cln1-/-* neurons (Kim et al., 2006). It has, however, later been shown by the same research group that ER stress leading to apoptosis is a common feature in many other lysosomal storage diseases, both neurodegenerative and non-neurodegenerative, and would thus not be specific to INCL (Wei et al., 2008). In line with the previous results concerning localized reactive gliosis, the (receptor for

advanced glycation end-products) RAGE signaling pathway has also been shown to be activated in astroglial cells, mediating pro-inflammatory cytokine production and contributing to the inflammation process (Saha et al., 2008).

In addition to the connection to defects in cholesterol metabolism by gene expression analysis, recent results (Ahtiainen et al. 2007; Lyly et al., 2008) demonstrate an increase in the cholesterol biosynthesis and a defective ApoAI metabolism in Ppt1 $^{\Delta ex4}$ mice.

2.3.7.2 CLN2

The activity of TppI has been abolished to create the mouse model for CLN2 (Sleat et al., 2004). The mouse resembles LINCL with a rapid neurodegenerative phenotype. Pathological characterisations have revealed a pronounced degradation of the thalamo-cortical system, with first symptoms appearing in the thalamus, alongside cerebellar atrophy (Sleat et al., 2004). A progressive reactive gliosis is evident in the motor cortex, hippocampus, striatum and cerebellum (Chang et al., 2008).

2.3.7.3 CLN3

JNCL is the most studied among NCLs with four different mouse models created to mimic the disease. The *Cln3-/-* mouse, which was generated by deleting exons 1-6 is the best characterised of them all (Mitchison et al., 1999). Other mice include *Cln3*$^{\Delta ex7-8}$ (Cotman et al., 2002), *Cln3* knock-out mouse (made by deleting exons 7-8 as the previous mouse model)(Katz et al., 1999) and the *Cln3* knock-in mouse, produced by deleting exons 1-8 with an extra ß-galactosidase reporter gene (Eliason et al., 2007).

All of the mice replicate the cellular pathology of JNCL with subunit c of the mitochondrial ATPase and fingerprint profiles, but the mice scarcely show any visible symptoms (Cooper et al., 2006).

Pathological analyses have demonstrated a selective loss of inhibitory interneurons (Mitchison et al., 1999). As in most other NCL-mice, the thalamo-cortical system is a target for neurodegeneration, beginning in the thalamus and preceeded by a low level of glial activation (Pontikis et al., 2005; Pontikis et al., 2004). However, recent studies claim that there is no actual neurodegeneration in the *Cln3* $^{\Delta ex7-8}$ with the observed neuron loss suggested to be age related (Herrmann et al., 2008). Pathological changes have also been reported in the optical nerve (Weimer et al., 2006). A specific unique feature for the *Cln3* deficient mouse is the presence of

autoantibodies, similar to JNCL disease (Chattopadhyay et al., 2002; Castaneda & Pearce, 2008).

Gene expression studies combined with molecular analyses have demonstrated changes in mitochondrial glucose metabolism, cytoskeleton, and synaptosome in the *Cln3-/-* mice (Luiro et al., 2006).

Studies on neuronal cultures from *Cln3*-/- mice have revealed defects in the endocytic pathway and in the transport and processing of Cathepsin D (the protein defected in CONCL). The neurons also show disturbed mitochondrial morphology and slight functional disturbances (Fossale et al., 2004; Luiro et al., 2006) The mice further suffer from selectively increased vulnerability to AMPA-type excitatory glutamate receptor toxcicity (Kovacs et al,. 2006; Kovacs & Pearce, 2008).

2.3.7.4 CLN6

There exists a natural mouse model for vLINCL, the *nclf*-mouse. The neurodegenerative phenotype resembles that of human patients (Gao et al., 2002; Bronson et al., 1998; Wheeler et al., 2002), with myelination defects, neurodegeneration, neuromuscular defects and progressive retinal atrophy and gliosis. Results from lipid analyses show accumulation of gangliosides GM2 and GM3 (Jabs et al., 2008).

2.3.7.5 CLN8

The motor neuron degeneration (*mnd*) mouse is a naturally occurring mouse strain, modelling EPMR disease (Ranta et al., 1999). It has a progressive neurodegenerative phenotype, which resembles more the severe vLINCL disease than EPMR disease, with retinal degeneration and severe paralysis. However, no epileptic seizures are evident in the *mnd* mouse (Messer et al., 1993; Messer et al., 1995). Even though this is the case, the mouse still replicates the changes in lipid metabolism, seen in EPMR patients (Vance et al., 1997). Increased concentrations of oxiradicals and lipid peroxides have also been found in *mnd* mice (Bertamini et al., 2002; Guarneri et al., 2004).

Some results have suggested that alterations in the glutamaergic neurotransmission may contribute to the disease in the mice (Battaglioli et al., 1993; Mennini et al., 1998; Mennini et al., 2002).

2.3.7.6 CLN10

The *Cathepsin D (Ctsd)* knock-out mouse is made by disruption of exon 4. The mouse presents a very grave phenotype, with the visceral organs affected in addition to the CNS (Saftig et al., 1995). The *Ctsd*-/- mouse only lives for 26 ±1days and it suffers from severe seizures. The seizures are connected to impaired GABAergic neurotransmission and reduce amount of GABA in the hippocampus (Shimizu et al., 2005; Saftig et al., 1995).

The pathological changes are pronounced in the thalamo-cortical system, and as in other NCL-models, starting in the thalamus and subsequently moving to the target neurons in the cortex. Reactive gliosis and microglial activation is also evident. Further pathological changes in the *Ctsd*-/- mouse include loss of neurons and synapses along with axonal degeneration (Partanen et al., 2008).

Additional studies have revealed that autophagic stress within the cells is evident, appears early on and increases with age (Koike et al., 2000; Koike et al., 2003). Apoptotic cell death has also been suggested as a mechanism for neurodegeneration in *Ctsd* deficient mice (Koike et al., 2003; Minarowska et al., 2007).

	Human gene	Mouse	Onset of symptoms	Phenotype	Neuropathology	Other characteristics
CONCL	Cathepsin D	Ctsd-/- knockout	3 weeks	Severe seizures, blindness, anorexia	Thalamocortical atrophy, progressive gliosis, axonal degeneration	Impaired GABAergic neurotransmission, accumulation of GM2 and GM3, abnormal lysophospholipid, apoptotic cell death
INCL	CLN1	$Ppt^{\Delta ex4}$ knockout	4 months	Loss of vision, motor abnormalities, seizures	Thalamocortical atrophy, progressive gliosis	Synaptic abnormalities, slight changes in Ca, early changes in neuronal cholesterol biosynthesis
	CLN1	Ppt-/- knockout	2 months			
LINCL and variants	CLN2	$Tpp1\ neo^{ins}$ Arg446His knock-out	9 weeks	Constant tremor, motor abnormalities, ataxia	Thalamocortical atrophy, cerebellar atrophy, progressive gliosis	n.d.
	CLN6	$Cln6^{nclf}$ spontaneous mutant	8 months	Motor dysfunctions, spastic limb paresis	Retinal atrophy	Accumulation of GM2 and GM3, reduced expression of GABAA2
	CLN8	$Cln8^{mnd}$ spontaneous mutant	4 months	Severe paralysis	Retinal atrophy	Changes in lipid metabolism, alterations in glutamatergic neurotransmission
JNCL	CLN3	Cln3-/- knock-out	16 months	n.d.	Late-onset thalamocortical atrophy, early low level glial activation, optic nerve degeneration	Vulnerability to glutamate receptor overactivation, mild mitochondrial, synaptic and cytoskeletal abnormalities, autoimmune response, autophagy
	CLN3	$Cln3^{\Delta ex7/8}$ knock-in	10-12 months			
	CLN3	Cln3 knock-out	14 weeks			
	CLN3	Cln3 del ex1-8, ins β-gal	2 months	Motor dysfunctions, reduced nocturnal activity, tremors, increased sensitivity to PTZ-induced seizures	n.d	n.d.

Table 3. NCL mouse models, adapted from (Cooper et al., 2006; Jalanko & Braulke, 2008). n.d. = not determined

3 AIMS OF THE STUDY

Prior to this study, both the clinical features of $vLINCL_{Fin}$ patients and the *CLN5* gene and disease causing mutations had been characterized. The CLN5 protein had been initially analysed but its function remained elusive. Consequently, the disease mechanism behind $vLINCL_{Fin}$ was unknown. Similar pathological features had been discovered in many NCLs, but no knowledge existed about common disease mechanisms and only initial connections had been observed between the affected proteins.

The aims addressed in this study were as follow:

- To analyse the brain pathology of $vLINCL_{Fin}$ utilising the novel *Cln5-/-* mouse model
- To analyse changes in gene expression in the novel *Cln5-/-* mouse model
- To discover possible common affected molecular pathways in *Cln5* and *Cln1* deficient mice.
- To discover molecular interaction partners for *Cln5*

4 MATERIALS AND METHODS

Materials and methods used in this thesis are described in the original publications.

Materials and Methods	Original publication
Immunohistochemistry	**I, II, III**
Confocal microscopy	**III, IV**
Cortical thickness measurements	**II**
Counts on neuronal number	**II**
Culturing of COS-1 and HeLa cells	**IV**
Dissection and culturing of primary neurons	**III**
Gene expression pathway analysis	**I, III**
Gene expression profiling	**I, III**
Histological processing of mouse brain	**II**
In vitro binding assay (GST pulldown)	**IV**
Light microscopy	**I, II, III**
Locus specific gene expression analysis	**III**
Mouse brain lysate preparation	**III**
Mouse brain tissue preparation	**I, II, III, IV**
Protein detection by Western blot analysis	**III**
Protein detection by immunofluorescence	**III, IV**
Quantitative thresholding image analysis	**II, III**
Real-time PCR	**I**
Regional volume measurements	**II**
RNA extraction	**I,III**
Transient transfections	**III,IV**

4.1 Animals

Homozygous mutant *Cln5*-/- and *Cln1*-/- mice were generated on a mixed C57BL/6Jx129SvEv strain background. The genotypes of all mice were determined by polymerase chain reaction of DNA from tail biopsies (Jalanko et al., 2005; Kopra et al., 2004).

All animal experiments were conducted in accordance with international standards on animal welfare and with approved animal policies of the National Public Health Institute, Helsinki, with adequate measures taken to minimize pain or discomfort.

5 RESULTS AND DISCUSSION

5.1 The *Cln5*-/- mouse (I)

Prior to this study, vLINCL$_{Fin}$ had only been studied in human patients. The *CLN5* gene had been cloned and positioned on chromosome 13q21-q32. However, CLN5 turned out to be a protein without any known functions or homologs (Savukoski et al., 1998). It was later shown to be a lysosomal protein (Isosomppi et al., 2002; Vesa & Peltonen, 2002), probably targeted to the lysosomes via the M6-P receptor pathway (Sleat et al., 2005; Sleat et al., 2007) Yet some uncertainty existed as to whether it is a soluble or transmembrane protein (Isosomppi et al., 2002; Vesa & Peltonen, 2002; Kyttala et al., 2006). As is the case with other NCL proteins, CLN5/Cln5 was proposed to have an additional function in the brain, due to the fact that in neuronal cells Cln5 appeared to additionally localize outside lysosomes in the neuronal cell soma and axons (Holmberg et al., 2004).

Atrophy of both cerebellum and cerebrum had been observed in autopsies of CLN5 patients (Tyynela et al., 1997). The cerebellar atrophy was rather severe, with almost complete destruction of the granule and Purkinje cells. Advanced loss of cortical neurons was also observed, with the remaining cells displaying pronounced storage enlargements.

These results were nevertheless only mirroring the end stages of the disease. In order to elucidate the specific function of Cln5 and the defected cellular pathways, a more complete view and knowledge of earlier disease events was crucial.

Mouse models for other NCL diseases were already available. The Cln1 (Gupta et al., 2001; Jalanko et al., 2005) Cln3 (Cotman et al., 2002; Katz et al., 1999; Mitchison et al., 1999), *nclf* (CLN6)(Bronson et al., 1998) and *mnd* (CLN8) (Bronson et al., 1993; Messer et al., 1993) mice had all faithfully replicated the cellular phenotype of their human disease counterparts.

In view of these results, it was meaningful to create a mouse model for vLINCL$_{Fin}$ in order to study the molecular and cellular mechanisms of this disease in greater depth.

The *Cln5* knock-out mouse was generated by targeted deletion of exon 3 of the mouse *Cln5* gene. The neomycin cassette inserted in exon 3, causes a frameshift mutation resulting in a premature stop codon. As a result only a truncated Cln5 protein is predicted to be synthesized in the cells. It is however likely that the truncated protein is eliminated by nonsense-mediated decay (NMD), a cellular

mechanism that limits the synthesis of abnormal proteins in the cell (Holbrook et al., 2004). The introduced mutation closely resembles the disease mutation found in a Swedish patient Glu253Stop. The homozygous mice did not show any birth anomalies and were born at a Mendelian frequency. The mice were fertile. The genotypes of all mice used in the subsequent experiments were determined by PCR.

5.1.1 The *Cln5*-/- mouse mimics the vLINCL disease in man

The *Cln5*-/- mice were followed from 5 weeks to 8 months of age and compared to their heterozygote and wild type (wt) littermates, to assess changes in their phenotype.

The visual progression of the *Cln5*-/- mice was followed by a forelimb extension test. The forelimb-extension test is thought to measure visual ability, but it is a common method to detect severe cognitive dysfunction (Mangiarini et al., 1996). A progressive impairment in this test was evident around week 13 and severe loss of vision could be detected at the average of week 21. Wt and heterozygous mice showed no changes in vision (**I**; figure 2). This result was consistent to the loss of vision in human patients.

In addition to the severe loss of vision, human patients suffer from other symptoms, such as epileptic seizures or motor abnormalities. Despite following *Cln5*-/- mice up to 8 months no other symptoms characteristic for human patients, could be detected.

After the initial phenotypic characterization, the *Cln5*-/- mice underwent neuropathological assessments. The typical neuropathological finding in all NCLs is the accumulation of autofluorescent material in the CNS. The major storage component in $vLINCL_{Fin}$ patients is subunit C of the mitochondrial ATP synthase complex and the ultrastructure of this storage material consists of rectilinear, curvilinear and fingerprint profiles. Therefore the *Cln5*-/- mice were assessed for accumulation of autofluorescent lipopigments.

6 month old *Cln5*-/- mice exhibited prominent autofluorescence throughout the brain. The autofluorescent material was scattered throughout the whole cortex, except for lamina I. Storage material was also evident in the retina, hippocampus and in some nuclei of the midbrain. The autofluorescence was nevertheless not as obvious in the cerebellum. Elevated autofluorescence was already observed in 1-month old animals; however, the amounts were distinctly less than in the older animals (**I**; figure 3).

Electron microscopic analysis was also preformed on brain sections of 3 month old mice, to be able to assess the ultrastructure of the storage material. Inclusion bodies were found in neurons of both cortex and thalamus, although not abundantly. They showed a mixture of lamellar bodies and both curvilinear and fingerprint profiles (**I**; figure 4). These results confirmed that the *Cln5*-/- mouse represents the human $vLINCL_{Fin}$ disease accurately, with visual failure /cognitive dysfunction and typical ultrastructural findings. What is notable is that the cerebellum which is severely affected in human patients is relatively spared in the mouse model, with the cortex and thalamus appearing to be predominantly affected.

Earlier studies on NCL patients, in conjunction with animal models, have shown evidence for progressive loss of GABAergic interneurons (Braak and Goebel 1978, 1979; Cooper et al., 1999; Cooper, 2003; March 1995; Walkley and March 1993; Williams et al., 1977). Immunohistochemical stainings on 1- and 6- month old *Cln5*-/- mouse brains were preformed to assess possible neuron loss and to address the nature of the degenerated cell type. Paraffin sections were stained with parvalbumin and calbindin, both markers for a subset of GABAergic interneurons. Compared to wt controls, a severe loss of both parvalbumin and calbindin positive interneurons were evident in the 6-month old *Cln5*-/- mouse (**I**; figure 5). The neuron loss was evident in several brain areas, including cortex, hippocampus, thalamus, midbrain and cerebellum. Within the cortex, the deeper laminae and entorhinal cortex were especially afflected by neuron loss. Loss of Purkinje cells was additionally evident in the cerebellum. Small changes in the immunoreactivity of both cortex and cerebellum could already be observed in 1- month old mice (**I**; figure 6).

These findings have been replicated in many NCL mouse models including; Cln1, Cln3, Cln8 and Cln10, (Bible et al., 2004; Cooper et al., 1999; Jalanko et al., 2005; Pontikis et al., 2004; Pontikis et al., 2005; Shimizu et al., 2005). In *Ctsd*-/- mice, the reason for this specific vulnerability of GABAergic interneurons might be due to reduced GABA levels, resulting from increased lysosomal degradation of GAD67. GAD67 is an enzyme that normally participates in the synthesis of GABA (Shimizu et al., 2005). However, how this applies to the rest of the NCLs is as yet unknown. Remarkably, several lysosomal storage disorders have been reported to show loss of GABAergic interneurons and, therefore, this phenomenon is likely to reflect lysosomal dysfunction (Walkley et al., 2009).

5.1.2 The old Cln5-/- mouse suffers from neurodegeneration, inflammation and loss of myelin

No function has yet been assigned for Cln5 and nothing is known about any specific cellular mechanisms that result in $vLINCL_{Fin}$. To get a more detailed description of the progression of the disorder at the gene expression level, we preformed microarray analysis of the *Cln5*-/- cortex. As the forelimb extension test for visual symptoms appeared positive around week 13, we wanted to assess gene expression changes during the active disease process. Therefore 3- and 4.5- month old mice were chosen for this analysis. Wt littermates were used as controls. The gene expression analyses were performed using the Affymetrix microarrays (Murine Genome U74Av2), which enables the measurement of 14 000 known genes. To average out the variability between different mice, we pooled RNA from two mice /genotype/age. The raw data was analysed with the MAS 5.0 (Affymetrix) software. We chose the fold change of 2 as a cut-off point, which gave us 16 differentially expressed genes in the 3-month old dataset and 68 differentially expressed genes in the 4.5 month dataset (**I**; table 1).

Since the number of differentially regulated genes was so small in the 3.5 month data, we decided to combine the resulting gene lists for further analysis. This was to categorize the combined results according to their Gene Ontology (GO) classifications, using the publicly available Gene Ontology Tree Machine tool-kit (Ashburner et al., 2000). Again, due to the limited number of genes, we included gene expression changes smaller than 2-fold, based on our hypothesis that many small changes in a pathway, especially in the brain, can together result in large changes. This analysis yielded three major pathways or structural components with significantly more genes observed than expected; Inflammation/defence mechanisms, Components of myelin, Neurodegenerative pathway (Fig. 3, **I**; table 2). These results are in line with the knowledge about the actual disease process: Brain autopsies from $vLINCL_{Fin}$ patients reveal loss of myelin, neurodegeneration and inflammation.

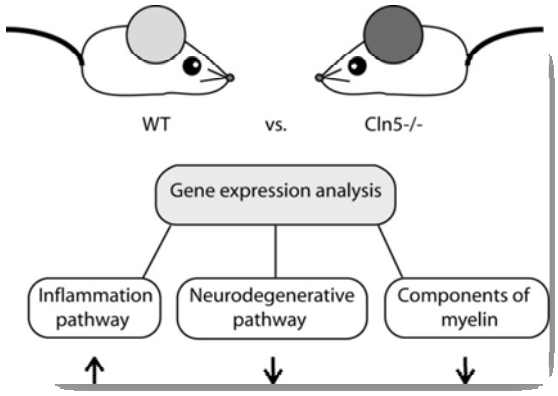

Figure 3. Affected pathways in the 6 month old *Cln5*-/- mouse

5.2 Histological characterization of the *Cln5*-/- mouse brain (III)

The function of the Cln5 protein remained elusive and except for the basic characterization of the *Cln5*-/- mouse, much was unknown about the brain pathology of the mouse. Many other NCL mouse models had already been histologically characterized, and a common theme seemed to evolve from this data. Detailed pathological analyses from *Cln1* (Bible et al., 2004), *Cln3* (Pontikis et al., 2004; Pontikis et al., 2005) and *Ctsd* (Partanen et al., 2008) deficient mice had highlighted the thalamo-cortical pathways as a starting point for NCL pathology. In these NCL-models neurodegeneration combined with reactive gliosis had been shown to initiate in the thalamus and subsequently move to the cortex. To gain a detailed view of the brain pathology of the *Cln5*-/- mouse we therefore performed stereological analyses of *Cln5*-/- mice at different stages of the disease. As previous results from other NCLs had highlighted the thalamo-cortical pathways, we focused our analysis on this part of the brain.

5.2.1. Neurodegeneration is initiated in the cortex of *Cln5*-/- mice

We first utilized 12 month old *Cln5*-/- mice and controls to analyze for progressive neurodegenerative changes in the cortex. Cavalieri estimates of **regional volume** revealed a widespread atrophy of the *Cln5*-/- brain. The affected areas included cortex, hippocampus, striatum and thalamus (**II**; figure 1A). No significant changes were, however, found in the volume of hypothalamus and cerebellum.

Late stage cortical atrophy is a common feature in all NCLs, both in humans and in mice. It is, however, mostly confined to specific regions (Bible et al., 2004; Kielar et al., 2007). In order to elucidate if any particular areas of the cortex were more severely affected than others, we made **thickness measurements** of different cortical regions, observing that the thinning was equal within all regions measured; primary motor cortex (M1), somatosensory barrelfield cortex (S1BF), primary visual cortex (V1) and late entorhinal cortex (Lent) (**II**; figure 1B). These results would suggest that cortical atrophy was widespread rather than regionally specific, providing evidence that the pathology of Cln5 differs in some way from other forms of NCLs.

Next, we wanted to investigate whether the observed thinning was uniform within all laminae of the cortex, by making a series of **lamina specific thickness measurements**. The results showed that the thinning was in fact lamina specific. Lamina V was affected through all measured cortical regions, while laminae IV and VI were affected in all regions bar M1. The rest of the measured laminae expressed a complex variability of changes in thickness; a reduced thickness within some areas, whilst an increased thickness in others. The increased thickness could be caused by "swollen" cells that have become enlarged due to large amounts of storage material within the cells. The changes in lamina thickness within different areas of the cortex can be viewed in Fig 4.

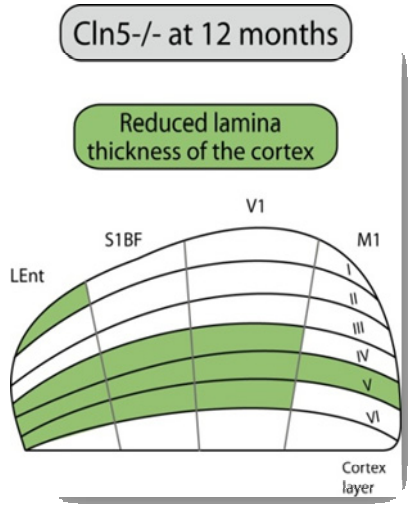

Figure 4. Lamina specific thickness in the cortex of 12 month old *Cln5* -/- mice

Since we had observed that both volume and thickness were reduced in the cortex of 12 month old *Cln5*-/- mice compared to controls, it was important to investigate possible changes in **neuronal number**. Other NCL mouse models had revealed that two specific cortical regions are affected by neuronal loss in the late stages of disease. These regions were S1BF and V1, the two target regions of the thalamic relay neurons that originate from ventral posterior thalamic nucleus (VPM/VPL) and dorsal lateral geniculate nucleus (LGNd), respectively. These thalamic areas were the ones that were initially affected in other NCL mice. We therefore focused our analysis on these two cortical regions (S1BF and V1), and to measure laminae IV-VI, which are the laminae that receive information or send feedback to the thalamus.

Unbiased optical fractionator estimates of Nissl stained granule neurons of 12 month old mice revealed a significant neuron loss in almost all regions analysed. Only lamina VI in S1BF did not show reduction of neuronal number at this age. We proceeded with our analyses, using 4 month old *Cln5*-/- mice. Interestingly, and contrary to all other NCL models, we witnessed a reduction of cortical neuronal number already by 4 months. There was a significant reduction of neuronal number in lamina V, evident in both S1BF and V1. The rest of the laminae in S1BF appeared to be spared, but a reduction of neuronal number was also evident in lamina IV of the visual cortex (V1) (Fig 5). This suggests that even though both areas of cortex are already affected by this early age, the neuron loss appears to occur earlier in the visual system. This relatively early cortical neuron loss differs from previous results from other NCL models, where cortical atrophy is evident only in late stages of the disease.

5.2.2. Thalamus is relatively spared

In the light of the previous observations regarding different NCL mouse models, we originally anticipated that there would be significant early neuron loss in the thalamus of the *Cln5*-/- mice. Until now it had been evident that the NCL pathology starts in the thalamus, subsequently spreading to the cortex. We used unbiased optical fractionator estimates of Nissl stained sections within three thalamic nuclei. These nuclei relay different sensory modalities to the cortex; the VPM/VPL which is connected to S1BF (somatosensory), the LGNd which is connected to V1 (visual) and the medial geniculate nucleus (MGN) which is connected to the auditory cortex. Again, contrary to our hypothesis there was no evidence of early neuron loss in the thalamus. There was significant loss of neurons in the VPM/VPL and the LGNd, but it was not evident before the age of 12 months, whereas the MGN seemed to be completely spared (Fig 5). These results suggest a reversed timing of pathological

events in the *Cln5*-/- mouse, compared to other NCL models. In the *Cln5*-/- mouse neurodegeneration occurs first within the cortex and only subsequently in the thalamus. It is known that lamina V of the S1BF projects to other subcortical structures, whereas lamina IV receives most of the imput from the thalamus and lamina VI projects back to the thalamus (Kandel et al., 2000). It appears that the neurodegeneration is initiated in lamina V, therefore possibly affecting the other cortical layers by interfering with the intercortical projections. However, the next lamina to be affected is lamina IV, receiving information from the thalamus. One possibility is that the neuronal loss in lamina IV is due to thalamus mediated events. This is supported by MEG recordings in human $vLINCL_{Fin}$ patients indicating inbalance between excitation and inhibition (Lauronen et al. 2002).

Previous data have shown that CNS expression patterns are similar for Cln1 and Cln5 (Heinonen et al., 2000b). Moreover, we have shown that both Cln1 and Cln5 share the same affected cellular pathway (see **III** and chapter 5.3 in this thesis). Furthermore our recent results have revealed that Cln1 and Cln5 interact with each other (see **IV** and chapter 5.4 in this thesis). Future studies of the actions of these two connected proteins should provide answers as to why they affect the thalamocortical system so differently.

5.2.3 The glial responses become evident before neurodegeneration

As mentioned earlier, mouse models of NCL share several common pathological features that include thalamo-cortical atrophy and pronounced early gliosis. Mouse models of NCL have been shown to display localized and progressive glial activation, which usually precedes neurodegeneration (Cooper et al., 2006). This reactive gliosis has been pronounced within the thalamo-cortical pathways. To determine if *Cln5*-/- mice share this phenotype with other NCL-mouse models, we stained sections from 1-, 4- and 12- month old mice and controls. Since reactive gliosis is characterized by hypertrophy of astrocytes and by proliferation of microglial cells and astrocytes, we imunostained brain sections of *Cln5*-/- and control mice for both of these cell types. For astroglia, we utilized the well characterized glial fibrillary astrocytic protein (GFAP), a member of the class III intermediate filament protein family, expressed in astrocytes and certain other astroglia in the central nervous system (Reeves et al., 1989). For microglial staining, we used the F4/80 protein, a cell surface glycoprotein that is a member of the EGF TM7 family of proteins, expressed on a wide range of mature tissue macrophages (McKnight & Gordon, 1998). We performed quantitative thresholding image

analysis for immunostainings of the thalamus, the S1BF and V1 regions of the cortex of *Cln5*-/- mice.

Although of a small magnitude, upregulation of GFAP immunoreactivity was already significant in the VPM/VPL nucleus of the thalamus and S1BF of the cortex of 1 month old *Cln5*-/- mice. The staining became more pronounced with increased age. At 4 months *Cln5*-/- mice displayed prominent GFAP immunoreactivity in the VPM/VPL nucleus of the thalamus and within all laminae of both the S1BF and V1 areas of the cortex, except for lamina IV. However, it was notable that the staining was more intense in the thalamus. At 12 months of age, both VPM/VPL and LGNd nuclei of the thalamus revealed intense immunoreactivity for GFAP, as did all laminae of both the S1BF and V1 of the cortex (**II**; figure 5)(Fig 5).

The quantitative threshold image analysis on F4/80 stained sections revealed no specific staining compared to controls in 1 or 4 month old *Cln5*-/- mice. However at 12 months of age a pronounced statistically significant staining could be seen in both the cortex (S1BF) and the thalamus (VPM/VPL) of *Cln5*-/- mice (**II**; figure 6).

Reactive gliosis is usually interpreted as a response to neurodegeneration. An alternative hypothesis implicates a protective role for GFAP upregulation as a response to very early changes, prior to neuronal degeneration, such as, supporting neurons by exporting glucose to them, minimizing neuronal damage by regulating harmful glutamate levels, mediating repair, promoting remyelinisation as well as regulating neurogenesis during development (reviewed in (Carmen et al., 2007; Liberto et al., 2004). Early glial responses have been evident in multiple forms of NCLs (Bible et al., 2004; Kielar et al., 2007; Oswald et al., 2005; Pontikis et al., 2005). In other NCL models this early gliosis occurs in the same areas of the brain as where the neuron loss subsequently takes place. However, this is not the case in *Cln5*-/- mice. The neuron loss starts in the cortex and can only later be detected in the thalamus at 12 months of age. Contrary, the reactive gliosis is evident in the thalamus already at the age of 1 month; months before any neuron loss can be observed. This data suggests that there could be some defects in the interplay between astrocytes and neurons, consistent with the suggestions made for other NCLs that imply protective glial responses occurring prior to neurodegeneration. Although this is plausible, we cannot rule out that the early astrocytic activation, unique for *Cln5*-/- mice, could be explained by other means, and may even be related to the function of Cln5 itself.

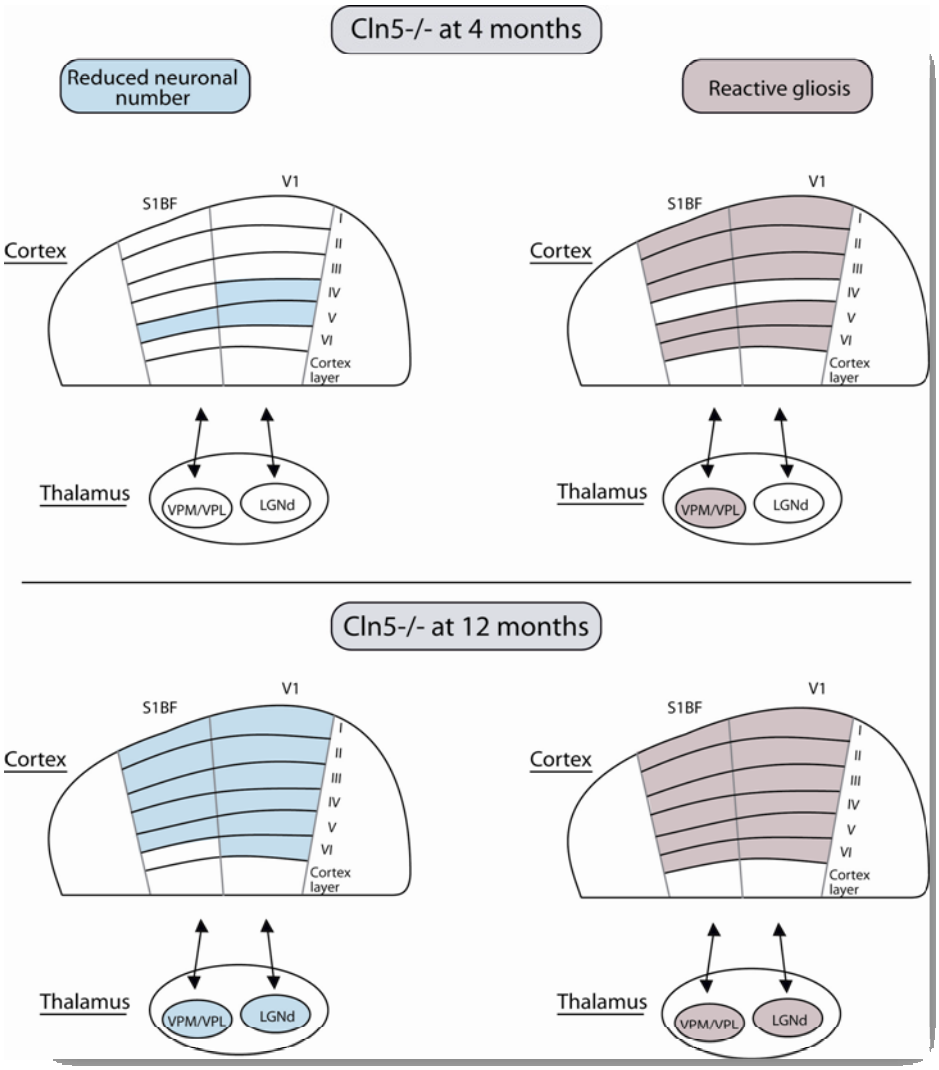

Figure 5. Reduced neuronal number and reactive gliosis in the *Cln5*-/- mouse brain

5.2.4 Loss of myelin occurs already during early stages of the disease

As loss of myelin is a general feature in vLINCL$_{Fin}$ patients (Holmberg et al., 2000; Rapola et al., 1999) we investigated if this feature manifests in *Cln5*-/- mice, by performing quantitative thresholding image analysis on Luxol fast blue (LFB) stained sections. LFB is an alcohol soluble stain, for the lipoproteins in the myelin sheath. Both Corpus callosum (cc) and cerebellum (cb) were measured. Significant loss of myelin in the corpus callosum could already be observed at the age of 1 month. The same loss could also be seen at the age of 4 months. An even greater loss of myelin was evident in the cerebellum at the age of 1 month, and the LFB staining was upregulated at the age of 4 months in the same region. This is most likely due to the fact that LFB stains for lipids, and severe lipofuscin storage can be observed in the *Cln5-/-* brain at the age of 4 months (unpublished data)(Fig 6).

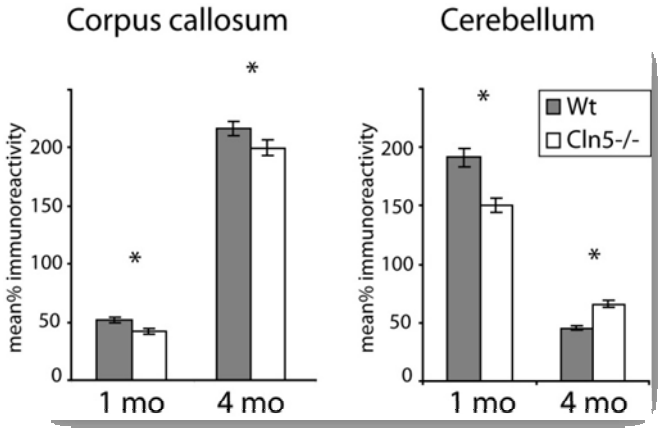

Figure 6. Changes in the myelin staining in the *Cln5*-/- mouse compared to wildtype (WT)

These results are in line with previous findings from vLINCL$_{Fin}$ patients. They are also consistent with our results from gene expression proliling analyses of *Cln5-/-* mice, that show that the expression of many genes connected to myelinization are downregulated (see 5.1.2 and 5.3.1). The loss of myelin is apparent already at the age of 1 month. Earlier time-points were not included in the original experimental design, so it is not known whether the reduced LFB staining reflects the loss of pre-existing myelin, or if the whole myelinization process itself is faulted and therefore delayed. The loss of myelin is concurrent with the emerging reactive gliosis in the 1 month old *Cln5-/-* mouse brains, potentially implying a connection between these two events. It has previously been shown that reactive astrocytes promote remyelinization in the brain (Carmen et al., 2007; Liberto et al., 2004). Additionally,

patients with Alexander disease, which is caused by mutations in the GFAP protein, also suffer from hypomyelinization (Li et al., 2002). Therefore, this could present another explanation for early astrocytic activation in the *Cln5* deficient brain; the reactive gliosis at this point could be a secondary event resulting from loss of myelin. The role of myelin and the myelin producing oligodendrocytes seems to be emerging behind NCL pathology. Magnetic imaging analyses of NCL patients with varied types of NCL disorders, have implicated loss of myelinization (Haltia 2003). Furthermore, a recent MRI study of several NCL mouse models also implicated major changes in the white matter, suggesting a prominent role of myelin related events in NCL (Haapanen et al., manuscript in preparation).

5.3 Cln5 shares a common molecular pathway with Cln1 (II)

Despite the identification of many genes mutated in NCLs, there were still many unanswered questions. How do mutations in so many different genes result in clinically and histopathologically similar diseases? Why is the CNS particularly vulnerable to disease? Is there a common defected pathway behind NCLs, resulting in similar clinical findings? To find answers to some of these questions, we performed gene expression profiling analyses on both *Cln5*-/- and *Cln1*-/- mouse models. The *Cln1*-/- mouse had also been generated by our laboratory (Jalanko et al., 2005) and represented a mouse model for INCL, the infantile form of NCL. Initial gene expression profiling analyses of both of the models had already been performed **(I**, (Jalanko et al., 2005)), but those analyses concentrated on time points representing later stages of the disease. Six month old *Cln1*-/- mice exhibited gross upregulation of inflammation related genes and 3 and 4.5 month old *Cln5* -/- mice displayed changes in genes that where related to inflammation, myelin integrity and neuronal degeneration. To identify early and progressive changes in genes and metabolic pathways that may contribute to the neurological phenotype observed in the *Cln1*-/- and *Cln5*-/- mice we chose 1 and 4 month old mice for our present analysis and used RNA from the cortex. We used the Affymetrix MOE 430A gene chips representing close to 14,500 annotated genes and more than 4300 ESTs.

We initially analyzed both mouse models separately, in order to find gene expression changes that were specific to each disorder.

5.3.1 Gene expression changes in the *Cln5*-/- mouse

Analysis of the 1 month old *Cln5*-/- mouse revealed 76 upregulated genes and 206 downregulated genes. The most upregulated gene was adenylate cyclase-associated protein 1 (*Cap1*, 3.9-fold) and the most downregulated gene was chemokine (C-C motif) ligand 21 a (*Ccl21a,* -1,7 - fold).

At 4 months, 34 upregulated and 80 downregulated genes were identified. The most prominent change was seen in the kinesin family member 5C (*Kif5c*), which was -6.4-fold downregulated. Other genes that were downregulated by greater than 5-fold, included DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 (*Ddx6*) and G-protein-coupled receptor family C, group 5, member B (*Gprc5b*). The most upregulated gene was dentatorubral-pallidoluysian atrophy protein (*Drpla*) with a fold change of 4.1.

Single gene changes are not however, usually as informative as the analysis of whole biological pathways. We therefore proceeded to analyze these using a custom made non-parametric pathway analysis algorithm. In the 1 month old *Cln5*-/- mice we discovered that pathways related to regulation of cell adhesion, RNA processing and transcription and phosphorylation were affected. Pathways related to myelination and camera eye development were affected in the 4 month old *Cln5*-/- mice (**III**; figure 2). These results were expected because $vLINCL_{Fin}$ patients are known to suffer from loss of vision and hypomyelinization (Holmberg et al., 2000; Rapola et al., 1999). Our previous results from immunohistochemical studies and from gene expression profiling (**II**, **I**) also revealed that the *Cln5*-/- mouse is affected by loss of myelin. We have additionally shown that neuron loss, typical for NCLs, starts in the visual cortex (V1) in *Cln5*-/- mice at the age of 4 months (**II**). This corroborated our novel microarray results. We could therefore assume that the results from the 1 month old mice are, to a degree accurate and related to the ongoing disease process, although there is no simple way to connect these microarray results to any "visible" defects in the young presymptomatic mouse. Our previous results had revealed ongoing neuronal death in the *Cln5-/-* mouse brain, already at the age of 4 months (**II**). No gene expression changes were, however, evident which would point to any activated apoptotic or autophagic signal cascades in the *Cln5-/-* mouse brain, most likely due to the fact that apoptosis or autophagy might be only secondary events resulting from neuronal cell death initiated by other mechanisms.

5.3.2 Gene expression changes in the *Cln1*-/- mouse

The analysis of the 1 month old *Cln1-/-* mouse revealed 261 upregulated and 156 downregulated genes. The most upregulated gene was cytotoxic granule-associated RNA binding protein 1 (*Tia 1*), with a fold change of 1.6. The most downregulated genes included *Cln1* and protein tyrosine phosphatate receptor type F (*Ptprf*), with fold changes of -3.5 and -2.2, respectively. When analyzing the 4 month old *Cln1*-/- mice we discovered 137 upregulated and 27 downregulated genes. The most upregulated gene, with a 32-fold increase in expression was *Cap1*. Interestingly, *Cap1* was discovered to be the most upregulated gene also in 1 month old *Cln5*-/- mice. The astrocyte related *Gfap* was another prominently upregulated gene, with a fold change of 16.6. The most downregulated genes included inactive X specific transcripts (*Xist*), *Gprc5b*, and *Ptprf*, with fold changes of -7.0-, -3.7- and -3.3, respectively. *Ptprf* had already been implicated in 1 month old *Cln1*-/- mice, and this result suggested that this gene or protein might be important in the Cln1 pathology. We further noted that both *Gprc5b* and *Ptprf* were also affected in *Cln5* deficient mice, suggesting a connection between these two NCL disorders.

The pathway analysis of 1 month old *Cln*1 defected mice revealed affected pathways that were connected to neuronal entities, signal pathways related to phosphorylation and calcium ion homeostasis. The calcium homeostasis pathway was also significantly affected in 4 month old *Cln1-/-* mice, in addition to inflammation associated pathways. Affected categories at this age included also cytoskeleton and lysosomes (**III**; figure 2). Again it seems as though the data from the 4 month old mice accurately supports our previous observations. Inflammation is a common feature in all NCLs and since PPT is a lysosomal enzyme, its alteration would be expected to affect the function of lysosomes. Changes in transcripts encoding calcium binding proteins have previously been shown by gene expression profiling of cultured *Cln1*-/- neurons. Further, functional studies revealed that *Cln1*-/- cells exhibited an improved ability to recover after a potentially excitotoxic stimulation with glutamate, as compared to wt cells (Ahtiainen et al., 2007).

5.3.3 Similar changes in gene expression suggest aberrations in a pathway regulating growth cone stabilisation

In order to search for common affected pathways between *Cln5*-/- and *Cln1*-/- mice, we compared both mouse models datasets to each other. We used gene lists of differentially expressed genes made separately for each model and each age group, comparing them so as to identify genes that showed expression changes in both of

the disease models. This comparison analysis revealed 51 genes that were altered in both *Cln5* and *Cln1* deficient mice (**III**; table 1). After extensive literature searches we identified several of these 51 genes to be critical in neuronal growth cone and cytoskeletal dynamics. We then went through the separate gene lists for each mouse model again, in order to find additional genes that could be linked to this affected pathway. As a result we revealed that many genes were differently expressed in the same pathway, in both *Cln5*-/- and *Cln1*-/- mice. Within this common pathway gene expression changes between the two mouse models could be identical, opposite, or only in one strain. The affected pathway is illustrated in Fig 7. Interestingly, several of the genes affected in both mouse models, especially those involved in phosphorylation, were found to be in a coexpressed gene cluster with Ppt1, the gene disrupted in the *Cln1*-/- mouse (**III**; figure 3). Several studies have implicated that coexpressed gene clusters represent genes potentially operating in the same metabolic pathway (Pal & Hurst, 2004), thus supporting the important role of receptor phosphorylation in both Cln1 and Cln5 mediated functions.

The combined analysis especially highlighted the downregulation of LAR PTP, implicated in neurite outgrowth, modulation of synapse formation and synaptic vesicle release (Stryker & Johnson, 2007). Interestingly, PPT1 expression is reported to be simultaneous with synaptogenesis (Isosomppi et al., 1999; Suopanki et al., 1999) and evidence of affected synaptic vesicles have been reported in the *Cln1*-/- mouse (Virmani et al., 2005). Additionally, we could detect upregulation of Src tyrosine kinase and the cyclase-associated protein Cap1. Thus, a link to the Src family kinase Fyn, which is implicated in several neurodegenerative conditions such as Alzheimer and Prion diseases (Lee 2005; Santuccione 2005), could be established. The gene expression profiling revealed a downstream linkage to the cytoskeleton, especially highlighted in the *Cln5*-/- mouse where we could demonstrate disturbed actin expression also at protein level. *Cln3*, the gene defected in JNCL, is also linked to cytoskeletal abnormalities (Luiro 2006). It will be interesting to determine if the cytoskeletal defects are common for all different NCLs, and wether they are responsible for deficiencies in the intracellular trafficking.

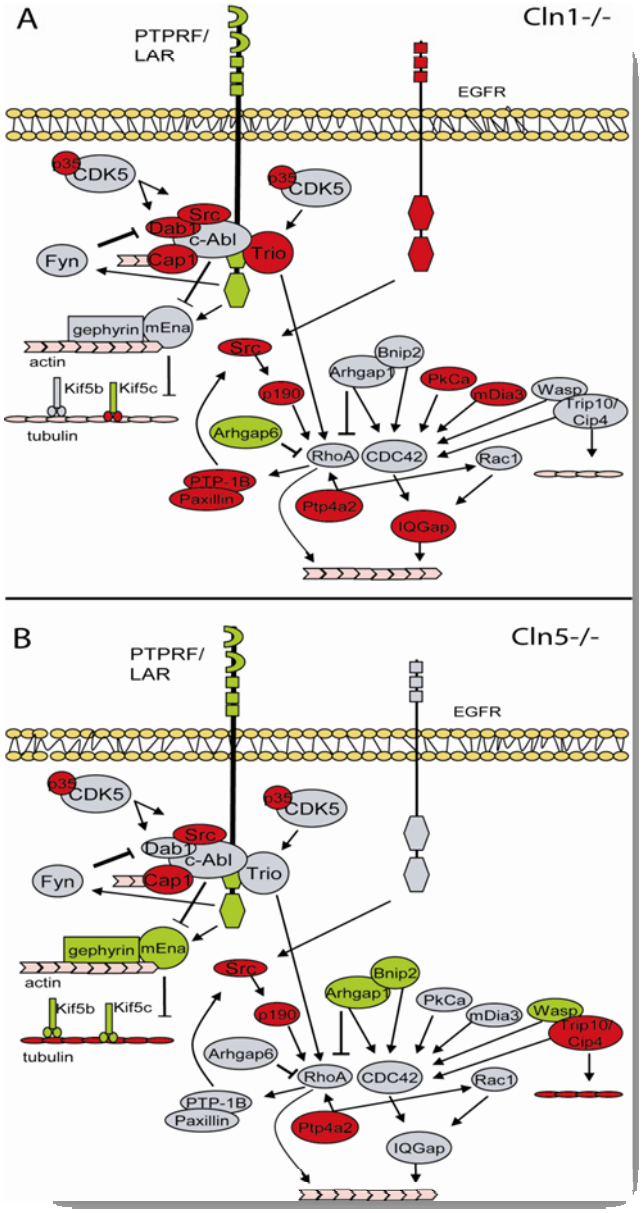

Figure 7.

A common affected pathway in *Cln5* and *Cln1* deficient mice. Upregulated genes are marked in red, whereas downregulated genes are marked in green. Genes with no difference in their expression are coloured gray.

Microarray methods are essentially for screening. They measure levels of messenger RNA (mRNA). Although it can not directly be assumed that there is a correlation between mRNA and protein levels, it might be reasonable to conclude that a

different expression level of a specific mRNA in a disease is an indication for some type of abnormality in eithe the encoded protein, an affiliated structure or function, and deserving of further investigation (Konradi C, 2005). However, it has to be noted that there are several limitations to microarrays. A major limitation is a decreased sensitivity of the arrays to detect genes with low expression levels. Another drawback is that microarrays do not measure posttranslational modifications, such as phosphorylation (Bunney et al., 2003). It must also be remembered, when working with the brain, that the brain tissue is heterogeneous containing many different nerve cells. This may decrease the sensitivity of microarrays by masking changes in gene expression. These issues should be kept in mind, when validating microarray data.

5.3.4 The cytoskeleton is affected

As our data from gene expression profiling implicated defects in several genes and pathways linked to the cytoskeleton, we further investigated the integrity of the cytoskeleton in the *Cln1* and *Cln5* deficient mice by analysing both localization and quantity of two known cytoskeletal markers; actin and tubulin. For this purpose, we preformed double immunofluorescence (IF) analysis on E15 primary cortical neurons from wt, *Cln5* and *Cln1* deficient mice. Although the gene expression profiling was performed on brain tissue, we used neuronal cell cultures, due to the fact that double immunofluorescence analysis techniques are more informative when performed on fixed cells.

The results from the *Cln1*-/- neurons displayed a loss of both actin and ß-tubulin staining in the cell soma compared to wt, whereas the growth cones of the mutant mouse neurons displayed greater and distinct staining by actin than the wild-type neurons (**III**; figure 4A).

The immunofluorescence analysis of *Cln5*-/- neurons also revealed cytoskeletal defects. The actin staining was relatively more intense in both the cell soma and processes, when compared to wt neurons. No changes were, however, evident in the ß-tubulin staining of *Cln5* deficient neurons compared to wild-type (**III**; figure 4A).

To elucidate the intracellular protein levels and localizations of these proteins we preformed Western blot (WB) analysis on both soluble and membrane-bound intracellular fractions made from *Cln5*-/-, *Cln1*-/- and wt mice. The fractions were prepared from 4 month old mice brains and stained with markers for actin and tubulin. By fractionating the cell lysate we were able to assess whether the proteins of interest were in soluble forms, or bound to cellular membranes. No changes were

seen in the quantity of actin staining between the two models (data not shown). However, the ß-tubulin staining was almost completely absent in the *Cln5*-/- mouse (**III**; figure 5B). To analyse the *Cln5*-/- mouse further, we proceeded with immunohistochemical analyses on actin and tubulin stained *Cln5*-/- whole brain sections. The quantitative threshold image analysis of these sections revealed that the actin staining was significantly increased in 1 month old *Cln5*-/- mice, whereas the ß-tubulin staining was significantly decreased in 4 month old *Cln5*-/- mice (**III**; figure 4B). These results confirm our previous microarray results and prove that the cytoskeleton is indeed affected, particularly in *Cln5*, but also in *Cln1* deficient mice.

5.3.5 The growth-cone is affected

When comparing the gene expression profiling data of *Cln5*-/- and *Cln1*-/- deficient mice, a pathway connected to neuronal migration and the growth cone emerged. We had already noted that the cytoskeleton is affected resulting from expression changes in genes related to this pathway and further demonstrated both quantitative and qualitative changes in the amounts of intracellular membrane-bound and soluble cytoskeletal proteins. Next we analysed for possible aberrations in the intracellular staining of growth-cone related proteins using immunofluorescene staining and confocal microscopy. We chose three well known markers for growth-cone assembly; Gap-43, synapsin and Rab3 for our analysis. Rab3 was, in addition, shown to be differentially expressed in our microarray data. Gap-43 is expressed at high levels in neuronal growth cones during development and during axonal regeneration. Even though it is not essential for axonal outgrowth or growth cone formation *per se*, it is required at certain decision points, such as the optic chiasm. It is thought that Gap-43 serves to amplify pathfinding signals from the growth cone (Strittmatter et al., 1995). Synapsin is a synaptic vesicle-associated protein, participating in synapse formation, regulation of the synthesis of other synaptic vesicle proteins and promotion of neurotransmitter release (Qin S 2004). Rab3 is known to regulate the fusion of synaptic vesicles (Geppert et al., 1997). Rab3 and synapsin are known to interact. Synapsin stimulates Rab3a recruitment to synaptic vesicles, whereas Rab3 inhibits the binding of synapsin to actin (Giovedi et al., 2004).

When analysing the *Cln5*-/- mice an abnormal staining of synapsin was observed. In wt mice the synapsin staining was evenly distributed, whereas in *Cln5*-/- mice the

staining was dotted around the cell soma. No other irregularities were however seen in the IF staining of *Cln5*-/- mouse neurons (**III**; figure 5A).

Next we analysed the *Cln1*-/- neurons. No changes were observed in the synapsin staining in *Cln1*-/- mice. Conversley, both of the other markers appeared abnormal. The Gap-43 staining, which stains axonal varicosities in wt mice, displayed a pronounced staining pattern around the cell soma in *Cln1*-/- mice and was almost absent from the varicosities in these mice. Rab3 staining was unusually faint in *Cln1*-/- neurons compared to wt, suggesting reduced amounts of this protein (**III**; figure 5A).

To examine the intracellular levels of membrane-bound and soluble synapsin, Gap-43 and Rab3 we proceeded with WB analyses on both soluble and membrane-bound intracellular fractions from *Cln1*-/- and *Cln5*-/- mice. Although, IF staining of synapsin appeared abnormally distributed in *Cln5*-/- neurons, no detectable change was observed by WB in the intracellular protein levels of this marker. However, the WB analysis revealed Rab3 to be almost completely absent from the cytoplasmic fraction in *Cln5*-/- mouse brain lysates. Additionally, no changes were seen in the IF staining of Gap-43, whereas a more pronounced membrane localization of this marker was evident in WB analysis (**III**; figure 5B).

We confirmed our previous result of pronounced IF staining by Gap-43 around the *Cln1*-/- neuron cell soma through WB analysis. Additionally, Gap-43 was enriched in the membrane fraction of *Cln1*-/- brain lysates. The Rab3 protein staining appeared unchanged, even though the IF staining suggested a reduced amount of protein in the cells. However, although we had not seen any apparent changes in the synapsin IF staining, the WB analyses indicated that the amounts of synapsin were greatly reduced in the brain lysate from *Cln1*-/- mice (**III**; figure 5B).

These results are in line with our microarray results, indicating a functional defect in the growth cone. We demonstrated on a cellular level that both the cytoskeleton and the growth cone are indeed affected. Gap-43 is palmitoylated (Liu et al. 1994) and has been shown to be depalmitoylated by PPT1 *in vitro* (Cho et al., 2000). Abnormal accumulation of Gap-43 in the ER has previously been implicated in INCL (Kim et al., 2006). Here we showed that Gap-43 was abnormally accumulated in *Cln1*-/- mice in the neuronal cell membrane, and to the membrane fraction of brain cell lysates.

5.4 CLN5 interacts with CLN1, CLN2, CLN3, CLN6 and CLN8 (IV)

Our finding of common affected pathways in *Cln1*-/- and *Cln5*-/- mice supported the fact that phenotypically similar NCL diseases may be linked by molecular interactions. CLN5 has previously been shown to interact with CLN2 and CLN3, based on coimmunoprecipitation and in vitro binding assays (Vesa et al., 2002a), but the significance of this interaction has remained unknown. Furthermore, Bessa and colleagues had demonstrated that the expression of both CLN3 and CLN5 was reduced in CLN2 patients and that the expression of CLN3 is reduced in CLN5 patients (Bessa et al., 2006). Taken together, all this data is suggestive of a common function or pathway for the NCL proteins. Therefore, we wanted to analyse if CLN5 has more interction partners among the NCL proteins, and particularly if it interacts with CLN1.

5.4.1 The interaction between CLN5 and other NCL proteins

The possible interaction of CLN5 with other NCLs was investigated by GST pull-down experiments. A GST-Cln5 fusion protein expressing the full length mouse Cln5, but lacking the signal sequence (GST-Cln526-341), was used to pull down endogenous NCL proteins from both mouse brain extract and HeLa cell lysates. We additionally used overexpressed proteins from lysates of transiently transfected COS-1 cells, in order to be able to monitor for interactions that may go undetected endogenously due to the naturally low levels of NCL proteins. The analyses showed that in addition to CLN2 and CLN3, CLN5 does interact with CLN1/PPT1, CLN6 and CLN8. Another lysosomal protein LAMP-1 was used as a negative control, and did not show any binding to CLN5 (**IV**; figure 1).

5.4.2 The interaction between CLN5 and CLN1 is strong and occurs already in the ER

To assess possible functional links between CLN1/Cln1 and CLN5/Cln5 we compared the localization of PPT1 in both *Cln5*-/- and wt mouse fibroblasts and overexpressed them together in HeLa cells. The cells were transiently transfected and fluorescently labeled with PPT1 and /or CLN5 specific antibodies, in addition to organelle specific markers. No changes where, however, seen in the lysosomal localization of PPT1 in *CLN5* deficient fibroblast, or when overexpressed together with CLN5 in HeLa cells (**IV**; supplement 1).

We then wanted to see if defects in the transport of CLN5 would somehow affect the localization of PPT1. To investigate this, we created a trafficking deficient CLN5 construct (TD-CLN5) by inserting an intramolecular flag-tag (CLN5-flag330) into the protein. This causes the TD-CLN5 to remain in the ER. Coexpression of both wt PPT1 and the TD-CLN5 resulted in the retention of PPT1 in the ER, indicating that the interaction between CLN5 and PPT1 is strong and occurs already in the ER. As a control we overexpressed PPT1 together with the trafficking-deficient CLN3-flag434 construct. However, CLN3-flag434 did not affect the lysosomal trafficking of PPT1 (**IV**; figure 2).

The most common vLINCL$_{Fin}$ disease mutation is the CLN5$_{FinMajor}$ (Tyr392X), which results in a truncated polypeptide lacking 16 amino acids from the C-terminus (Savukoski et al., 1998). It has previously been shown that this mutation causes the CLN5 protein to be retained in the ER and Golgi compartment (Isosomppi et al., 2002). To evaluate if the interaction between CLN5$_{FinMajor}$ and PPT1 would affect the transport of PPT1, CLN5$_{FinMajor}$ and wt PPT1 were overexpressed together in HeLa cells. Intriguingly, we noticed that the normally ER/Golgi retained CLN5$_{FinMajor}$ was clearly localized to the lysosomes (fig 8, **IV**; figure 4). This indicates that the overexpressed PPT1 is still able to interact with mutated CLN5 and facilitate the transport of the mutated protein to its correct localization in the lysosomes. To verify this result we repeated the experiment also in human neuroblastoma cells (SH-SY5Y), where the same effect was detected (**IV**; supplement 2). Our recent, unpublished results by Schmiedt and Bessa have further revealed that overexpressed PPT1 is able to facilitate the correct transport of CLN5 proteins that carry all the other known vLINCL$_{Fin}$ disease mutations.

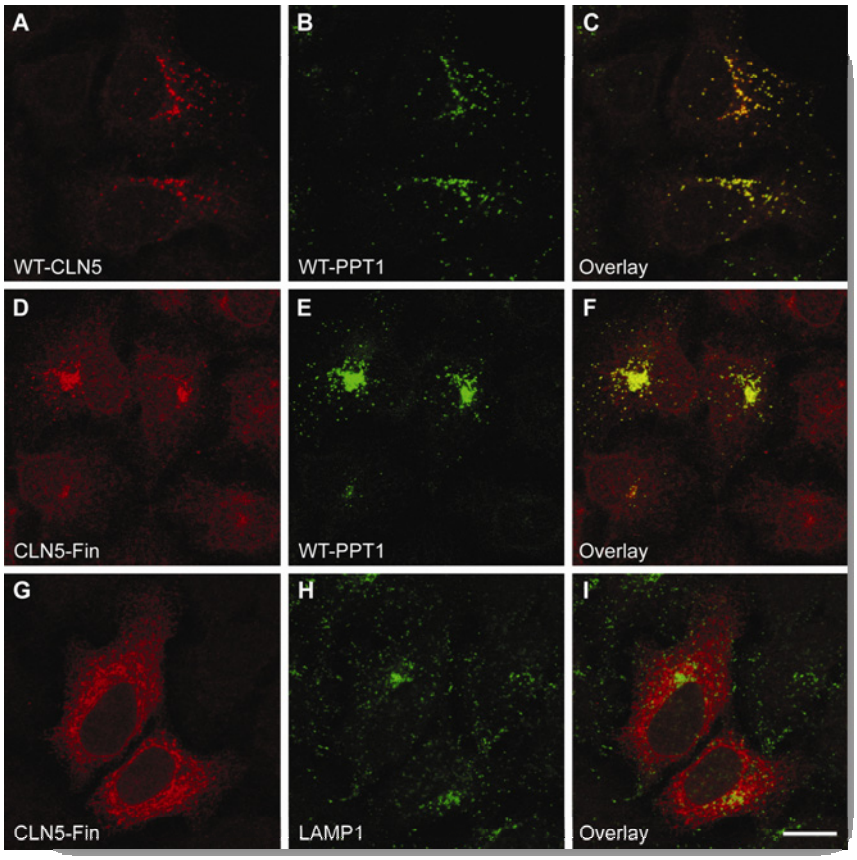

Figure 8. Co-expression of wt CLN5, $CLN5_{FinMajor}$ and wt PPT1/CLN1 in HeLa cells.WT-PPT is able to transport the mutated $Cln5_{FinMajor}$, normally residing in the ER, to the lysosomes.

5.4.3 Both CLN5 and CLN1 interact with F1-ATP synthase

Lyly and colleagues have previously shown that CLN1 interacts with the F1-complex of the mitochondrial ATP synthase (Lyly et al., 2008). In addition to its normal localisation in the mitochondria, F1- ATP synthase has been found on the surface of several different cell types (Kim et al., 2004; Martinez et al., 2003; Moser et al., 2001),where it can act as a receptor, for example for Apolipoprotein A-1 (Apo A-1). The amount of ectopic F1-complex (Alpha and beta subunits) were shown to

be incresed in *Cln1*-/- neurons, and consequently, Apo A-1 uptake was increased (Lyly et al., 2008), pointing to disturbances in cholesterol metabolism.

As we detected an interaction between CLN5 and PPT1, we wanted to investigate if CLN5 would also interact with the F1- ATP synthase. GST-CLN5 was used to pull down proteins from an enriched lysosomal/mitochondrial fraction prepared from mouse liver. We were able to show that CLN5 does interact with both α- and β-subunits of the F1-complex (**IV**; figure 7). In order to determine if the interaction between CLN5 and the F1-complex was mediated by PPT1, or vice versa, we repeated the experiment using cell lysates from both *Cln1* and *Cln5* deficient mice. We were able to show that both CLN5 and PPT1 were able to interact with the F1-complex without the presence of each other (**IV**; figure 7, fig 9), further strengthening our previous finding of the interaction between CLN5 and PPT1. This raises the question of whether there are also disturbances in cholesterol metabolism in the *Cln5* -/- mouse. Cholesterol is known to be an important component of myelin. Our observations of defects in the myelination of nerve fibers in the *Cln5*-/- mouse brain (see **I, II**) now link these findings.

5.4.4 The functional consequences of the interactions between CLN5 and CLN2, CLN3, CLN6 and CLN8 remain elusive

In our experiments we showed that CLN5 interacts with many other NCL proteins; CLN2, CLN3, CLN6 and CLN8 (**IV**; figure 1, fig 9). The interactions of CLN5 with CLN2 and CLN3 have been previously published (Vesa et al. 2002a). The reproduction of these results here validates the earlier results. Only the mature lysosomal form of the CLN2 protein was pulled down with GST-CLN5, and not the precursor, indicating that unlike the interaction with PPT1, the interaction between CLN5 and CLN2 does not occur until within the lysosomes or late endosomes.

Localiszation of all NCL interaction partners was studied in wt and *Cln5* deficient mouse fibroblasts, but as in the case of PPT1, no changes in locations could be detected (**IV**; supplement 1). All binding partners were also overexpressed together with the transport deficient TD-CLN5 construct in HeLa cells. TD-CLN5 had only a minor effect on trafficking of CLN3 (**IV**; figure 2), and did not affect the localization of other interaction partners (**IV**; figure 3), supporting our pull down data, that suggests that the interaction with CLN2 occurs after the proteins have left the ER. CLN6 and CLN8 are both known to be ER resident, so it is likely to assume that the interactions between these proteins and CLN5 occur in the ER. As the trafficking of CLN3 was partially affected by the simultaneous expression of TD-CLN5, it is possible that the two proteins interact already in the ER. Future studies

will help us to understand the consequences on the NCL pathology of the interactions in this CLN network.

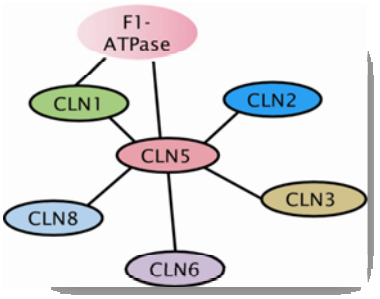

Figure 9. CLN5 interacts with CLN1, CLN2, CLN3, CLN6, CLN8 and with F1-ATPase

In summary, we have been able to show that CLN5 is connected to most other NCL proteins at the molecular level, suggesting a central role in the NCL interactome. Coimmunoprecipitation analysis would be a widely accepted method to verify the protein interactions but due to the lack of efficient antibodies the interactions were verified by GST-pulldown assays. GST-pulldown often requires overexpression of proteins to yield consistent results, as has been the case for most of our experiments. Furthermore, the cells have to be lysed and the proteins' physiological environment is therefore disrupted, making it possible for proteins to interact with partners that they never normally come into contact with. We have however, been able to show that our claimed interactions are plausible with regard to the interaction site, as shown by immunofluorescence analyses.

Other things to consider include possible protein misfolding, potentially leading to the exposure of hydrophobic surfaces and resulting in artificial interactions. This could be the case especially for CLN3, which is a transmembrane protein, known to be highly hydrophobic. On the other hand, CLN3 along with CLN2 have already previously been shown to interact with CLN5 by co-immunoprecipitation and in-vitro binding assays (Vesa et al., 2002). Furthermore, the restricted spatial environment within a GST-pulldown assay, having both small internal cavities in the agarose beads and the capability of GST to dimerise, might give rise to restricted mobility and therefore enhance the apparent affinity of an interaction. Therefore, careful controlling of the experimental conditions is necessary and we have used a hydrophobic lysosomal transmembrane protein LAMP-1 as a negative control to exclude non-specific interactions.

Yeast or mammalian two hybrid assay is an alternative method to GST-pulldown assay. The yeast two hybrid method was not utilized due to the fact that CLN5 does not have a homolog in the yeast and therefore the putative interactions may not be

present in the yeast environment. Mammalian two-hybrid assay is a relevant choice, but to our opinion it would not provide additional evidence as compared to GST pull-down technique since also this system utilizes overexpression of at least one interaction partner. In addition to the evidence of interactions obtained by the GST pull down assays, we feel that the identification of the common interaction partner for CLN1 and CLN5, the ATP-synthase, further provides a common funcitonal link to these two proteins and gives further support for the existence of the interactions.We have, also been able to confirm the alleged interaction between CLN5 and CLN1 on a functional level, which should reduce any concerns about the reliability of the interaction.

6 CONCLUSIONS AND FUTURE PROSPECTS

In the course of this study CLN5 has been shown to localize to the lysosomes and interact with two other NCL proteins, CLN2 and CLN3 (Isosomppi et al., 2002; Vesa & Peltonen, 2002; Vesa et al., 2002a). *CLN5* mutations have been found in patients outside northern Europe; in Columbia, Netherlands, Portugal and Newfoundland. Natural animal models with mutations in the *CLN5* gene have also been discovered. $vLINCL_{Fin}$ has been shown to exist in Border Collie dogs (Melville et al., 2005), Devon cattle (Houweling et al., 2006), and in Borderdale sheep (Frugier et al., 2008).

Many other NCL mouse models have been generated and analyzed resulting in many common features for NCLs being discovered. The thalamocortical pathways have been shown to be affected in all studied mouse models for NCL. From these studies we now know that neurodegeneration initiating from the thalamus and subsequently moving to the cortex, combined with reactive gliosis is a common feature in all NCLs (reviewed by (Cooper et al., 2006)). We also know that selective loss of interneurons is evident in all NCLs, although this may be a general feature of lysosomal storage disorders as a whole.

Due to the diligent efforts of Siintola and colleagues, we have recently been able to add two new NCL disease genes to this group. Mutations in the *MFSD8* (Siintola et al., 2007) gene result in the variant late CLN7 disease, whereas mutations in Cathepsin D (*Ctsd*) gives rise to the congenital CLN10 (Siintola et al., 2006b). These two additions have enlarged the group of known NCL genes to eight, and existing family materials implicate that more NCL genes are yet to be identified.

New interaction partners have been proposed for some of the NCL proteins. F1-ATPase interacting with CLN1/PPT1 (Lyly et al., 2008) as well as Hook1 (Luiro et al., 2004), Calsenilin (Chang et al., 2007), Na^+, K^+ ATPase and Fodrin (Uusi-Rauva et al., 2008) interacting with CLN3, have connected these proteins with potential novel functions.

We have focused our attention on the *Cln5*-/- mouse model, which has proven to be a valuable disease model for $vLINCL_{Fin}$. We started by analysing gene expression changes in the already symptomatic mouse, and were able to show that the mouse is affected by loss of myelin, inflammation and neurodegeneration related changes.

We continued, thereafter, with characterization of the brain pathology of the *Cln5*-/- mouse. We revealed that the *Cln5*-/- mouse shares the same thalamocortical pathology with other NCL mouse models, but the order of events were opposite to those of all the other NCLs. In most NCLs it is the thalamic relay neurons that die

first whilst the neurons in the cortical target regions die later. In *Cln5* deficient mice, the neurons die first in the cortex and only months later in the thalamus. We could similarly confirm that the same reactive glial responses are also present in our *Cln5*-/- mice, as in all other NCL mice. We were, however, again able to point out some important differences. It has been shown that the localized reactive gliosis is present early in the disease progression, in the areas where neuron loss is subsequently about to occur. In the *Cln5*-/- mouse, it appears that the early onset reactive gliosis not only occurs much earlier than in the other models, it also occurs more prominently in the thalamus, which is not the brain area where the neurodegeneration initiates in *Cln5*-/- mice. In agreement with our previous gene expression analyses that revealed a downregulation of myelin associated genes, we could show that significant loss of myelin stained fibres is already evident at 1 month of age.

We further performed gene expression studies on presymptomatic mice, as the early stages are most likely to pinpoint true dysregulated pathways. In these studies we included *Cln1*-/- mice into our analysis. Our hypothesis was that common changes in gene expression are likely in these two NCL mouse models, since the diseases are so alike. Indeed, we revealed a shared defected pathway in both mouse models. This pathway regulates the growth cone and cytoskeleton in neurons, and defects in it are known to affect neuronal migration. We were additionally able to show that several genes of this pathway are most probably coregulated with *CLN1*, suggesting similar functionality between these coregulated genes.

Based on our results for a shared pathway, and possibly similar functions, between *Cln5* and *Cln1*, we wanted to find out if these two genes might interact. Since some reports indicated interactions between a few NCL-proteins, we additionally included other known NCL genes into our analysis. We were able to demonstrateby GST-pulldown, interactions between CLN5 and CLN1, CLN2, CLN3, CLN6 and CLN8, connecting, for the first time, most of the known NCL genes to each other. Importantly, CLN5 seemed to represent a central molecule in this NCL interactome. The strongest interaction was found between CLN5 and CLN1. CLN1 had recently been shown to interact with the F1-ATPase, by surface plasmon resonance and GST-pulldown assays, so it was natural to investigate if CLN5 also interacted with it. Not surprisingly, we were able to show that CLN5 also interacts with the F1-ATPase. We further demonstrated that the overexpressed PPT1 is able to facilitate the transport of the mutated CLN5 protein to its correct localization in the lysosomes. This is not the first time that lysosomal proteins have been shown to assist each other in their transport to lysosomes. Both the ClC7 chloride transporter (Lange et al., 2006) and the lysosomal membrane protein LIMP-2 (Reczek et al., 2007) have been shown to assist the transport of their interaction parteners (OSTM1 and β-glucocerebrosidase, respectively). In the case of LIMP-2, it was shown that it

is required for a M6-P independent targeting of the β-glucocerebrosidase to the lysosomes and as is the case with CLN5 and PPT1, overexpression of LIMP-2 is able to facilitate the correct transport of β-glucocerebrosidase from the ER to the lysosomes (Reczek et al., 2007). It has been thought that both PPT1 and CLN5 would be targeted to the lysosomes via the M6-P dependent pathway (Sleat et al., 2006; Sleat et al., 2007). Our data suggest that there may be alternative trafficking routes to the lysosomes for these proteins.

This thesis has been able to shed light on the brain pathology of the *Cln5*-/- mouse and has led to the identification of several interaction partners for CLN5.

We now know that the brain pathology begins early in the *Cln5*-/- mouse, much earlier than any visible symptoms. It starts with loss of myelin and reactive gliosis. It is hard to say if the loss of myelin results from oligodendrocytes dying or from a developmental defect in the assembly of myelin. Since 1 month is the earliest time point we have investigated, we can not rule out that the defect in myelin emerges even earlier than this. Nevertheless, the first signs of neuron loss in the cortex appear around the age of 4 months and this dying of cortical target neurons most likely affects the thalamic relay neurons which start dying a few months later.

We have shown that these events are connected with disturbances of the cytoskeleton and the growth cone. Future studies will reveal, wether the cholesterol metabolism is altered in the *Cln5*-/- mouse, similarly to the *Cln1*-/- mouse, which also interacts with the F1-ATPase (Lyly et al., 2008). Cholesterol is a major lipid in the myelin sheath and it is possible that disturbances in the cholesterol metabolism would affect the assembly of the myelin sheath, leading to the loss of myelin we have observed in *Cln5*-/- mice. As all these different events can be connected to myelin disturbances, it would be of great interest to investigate if Cln5 harbours a specific role in oligodendrocytes. It might turn out that NCLs are diseases of neurodevelopment, rather than neurodegeneration.

The function of the Cln5 protein still remains elusive. Future studies will reveal whether Cln5 harbours a function already in the ER, as the proposed interactions with Cln1, Cln6 and Cln8 appear to transpire already there. On the other hand, the interaction between Cln5 and the F1-ATPase connects Cln5 with the mitochondria and the plasma membrane. However, increasing evidence has shown us that $vLINCL_{Fin}$ together with the rest of the NCLs may have to be considered in the future as other than purely lysosomal diseases. Regardless, the focus of the Cln5 recearch, should switch from neurons to encompass all brain cells, including astrocytes and oligodendrocytes

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